Insights into the physiology of the gamma-delta T Physiologie des lymphocytes T gamma-delta dans l'interaction du cytomégalovirus avec son hôte immunodéprimé

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ABREVIATIONS

AIM2, Absent In Melanoma 2
CD, cluster of differentiation
cDC , classic DC
CDR3, complementary-determining region 3
CMV, cytomegalovirus
DC, dendritic cell
DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
E, early
EGF, Epidermal Growth Factor
EGFR, Epidermal Growth Factor receptor
EPCR, endothelial protein C receptor
ER, endoplasmic reticulum
EVR, everolimus
FcR, Fc receptor
HCMV, human cytomegalovirus
HLA, human leucocyte antigen
HPC, haematopoietic progenitor cells
ICAM, InterCellular Adhesion Molecule
IE, immediate early
IFN, interferon
Ig, immunoglobulin
IL, interleukin
IRF, interferon-regulatory factor
LCMV, Lymphocytic choriomeningitis virus
LFA-1, Lymphocyte function-associated antigen
MPA, mycophenolic acid
mRNA, messenger RNA
mTOR, mammalian target of rapamycin
mTORi, inhibitor of mTOR
NK, natural killer
pAg, phospho-Antigen
pDC, plasmacytoid dendritic cell
PDGFR, Platelet-derived growth factor receptors
PI3K, Phosphoinositide 3-kinase
pp65, phosphoprotein 65
PRR, Pattern recognition receptors
TCR, T cell receptor
TGF, transforming growth factor
TLR, toll like receptor
TNF, tumor necrosis factor
UL, unique long
US, unique short
VCAM, Vascular Cell Adhesion Molecule

I. Objectives of the work

Cytomegalovirus (CMV) is still responsible for the most common opportunistic infection in immunocompromised hosts throughout the world (*1*). Latency after a first infection and the mode of CMV transmission contribute to its large prevalence. On the other hand, some of its viral characteristics which lead to its large cellular tropism contribute to its life-threatening impact among immunocompromised hosts during both primary infection, reactivation or superinfection as opposed for example to other herpesviruses. The symptoms associated with the direct and indirect effects of CMV closely depend on the type and quality of interactions between the host's defenses and the virus. In this work, we try to improve our understanding about the immune arsenal that operate for virus control and in particular among the γδ T cell compartment, one of the most recently studied actors of immune response to CMV. Secondly, we investigated the T cell response profile during viral reinfection in patients with a pre-established immune response. We attempt to understand why some patients might to be able to perform strong viral control compared to others, giving additional clues to help them restore a stronger host defense, especially with an immunosuppressive regimen including mTOR inhibitors (mTORi).

II. Viral characteristics of CMV

A. Herpes viral families and their host

The family *Herpesviridae* is classified into three subfamilies, the Alpha-, Beta- and *Gamma Herpesviridae*, which were originally defined by biological characteristics, but are now also seen as major lineages according to criteria of gene content and sequence similarities (*2, 3*). Fuller sequencing of seven mammalian herpesvirus genomes has shown that viruses from all three subfamilies contain a subset of around 40 genes that, by the criteria of genomic position

25   and similarities in encoded amino acid sequences, are common to all the viruses. From these

26   data and many single gene sequences for other mammalian and avian herpesviruses, it is clear

27   that these viruses have a common evolutionary origin (*4*).

28   To go further, eight well conserved genes (UL2, UL5, UL15, UL19, UL27, UL28, UL29 and

29   UL30) that are common to mammalian herpesviruses were used to elaborate phylogenetic

30   trees and the alignments of their sequences with pairwise divergence favor a common herpes

31   virus ancestry which confirms the three different α, β, and γ subfamilies (*5*).

32   Next, the phylogenetic tree of the alphavirus virus was performed using estimates of the rate

33   of change for the *a-herpesvirus* UL27 (gB protein) gene sequences, which was the most

34   studied. A time scale of events was achieved, based on the proposition that most lineages

35   arose through ancient cospeciation with hosts. A comparison was made between the tree

36   branching model for viruses of different host species with the branching of host trees and

37   between the ranges of divergence of viral gB sequence pairs and paleontological estimates of

38   times (*6*) (Figure 1).

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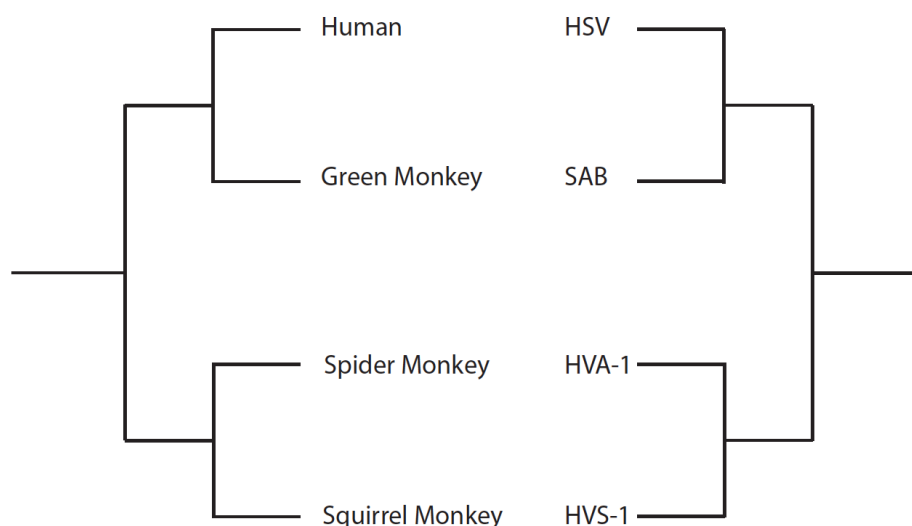
Figure 1 Comparison of host and alpha herpesviruses phylogenetic trees.

Phylogenetic trees of alpha-herpes viruses were constructed by alignments of 14 alphaherpesviral gB sequences.
Next, the branching pattern of the tree for viruses from different host species was compared to the branching of
the host's tree. (6)

45 Thus, an approximate proportionality between magnitudes of pairwise divergences of viral

46 sequences (substitutions/site in one lineage) and times since lineages of corresponding pairs

47 of hosts split lead to establish the timescale of alpha herpes evolution and constitutes the first

48 evidence that evolution was linked to host evolution (*6*). Assuming a constant and uniform

49 molecular clock for all subfamilies, estimating the rate of change by comparing the

50 differences for the dataset with those for the UL27 dataset made it possible to extrapolate a

51 time scale for the other subfamilies. The graph below (Figure 2) compares divergence values

52 for composite tree characteristics (from 20 species, Figure 2A, from 46 species, Figure 2B)

53 with possible equivalent dates in host evolution (*7*) in millions of years (My) before the

54 present (Figure 2C). For the α2 and β1 examples noted above, selected pairs of divergence

55 values for virus lineages present in the species tree were plotted (*i.e.,* those with the best

56 quantitative support) against host lineage divergence times (*4*). The straight line indicates an

57 overall consistency of pairwise divergences with cospeciation. Altogether, this high level of

58 congruence between the herpesvirus phylogenetic tree and that of the virus hosts' lineages

59 together with comparison between divergences for branch points in the herpesvirus tree and

60 dates of corresponding events in mammalian evolution indicates that cospeciation has been a

61 prominent feature in herpesvirus evolution (*5, 6, 8*). These observations make it clear that the

62 three subfamilies were born around 180-220 million years ago. The major siblings of the

63 subfamilies were probably generated prior to the mammalian radiation from 80 to 60 million

64 years ago, and these specimens within the siblings occurred over the previous 80 million

65 years (*5*). We will see later, in particular, that the beta herpesviruses have emerged in host that

66 already presented all the actors of immune system. Given the length of this genetic

67 relationship, it is highly likely that adaptations in both host immunity and viral genomes

68 enhance the host capacity to coexist with these old residents. These complex interactions

69   between virus and host form an intricate network of interdependent genes and processes that

70   we tried to understand in molecular detail below.

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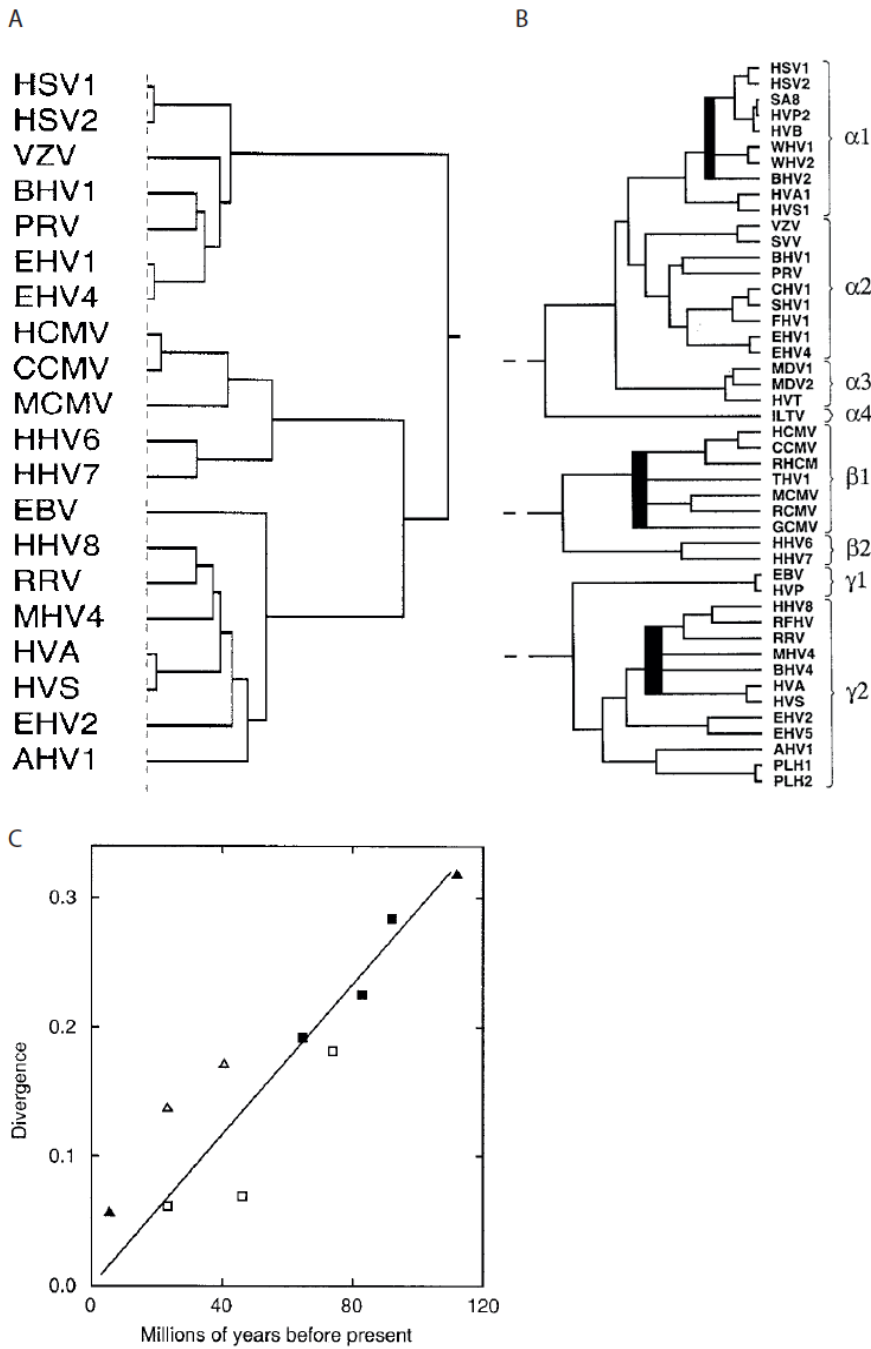
72   *Figure 2 Estimation of a correlation between the timescale of virus and of host.*

73   *Phylogenetic tree of 26-species of herpes families (A), of 46-species of herpes families (B) and comparison*
74   *between divergences for branch points in the herpesvirus tree and dates of corresponding events in mammalian*
75   *evolution (C).Figure 2C compares divergence values (substitutions/site in one lineage) features in the composite*
76   *tree for α2 sublineage (triangles) and β1 sublineage with dates of possible equivalents in host evolution (10) in*
77   *millions of years before the present (My). The host datings are from a recent analysis using DNA sequences of*
78   *vertebrate genes Filled symbols, data from 20-species phylogenetic tree (A); open symbols, data from 46-species*
79   *tree (B). The line was drawn through the origin and the four highest-value filled symbols. For example the*
80   *divergence events and times for humans/chimpanzees is 5.5 My (8).*

B. Viral structure and cycle

i) Viral structure

Human cytomegalovirus (HCMV) is a widespread beta human herpesvirus, also known as human herpes type 5. Compared to other human herpesviruses, HCMV is the largest, with a genome of 235 kb encoding 165 genes. The virion consists of a double-stranded linear DNA, an icosahedral nucleocapsid, enveloped by a proteinaceous matrix (the tegument). These components are enclosed in a lipid bilayer envelope. The tegument compartment contains the majority of the virion proteins, the most abundant of which is the lower matrix phosphoprotein 65 (pp65), also termed unique long 83 (UL83) (*9*). The tegument contains also some cellular and viral mRNA (*10*) and enzymes such as the DNA polymerase, the protein kinase and a cellular topoisomerase II, needed for viral replication (*11*). The host cell endoplasmic reticulum Golgi intermediate compartment-derived lipid bilayer envelope surrounding the tegument contains at least 20 virus-encoded glycoproteins that are involved in host-cell attachment and entry.
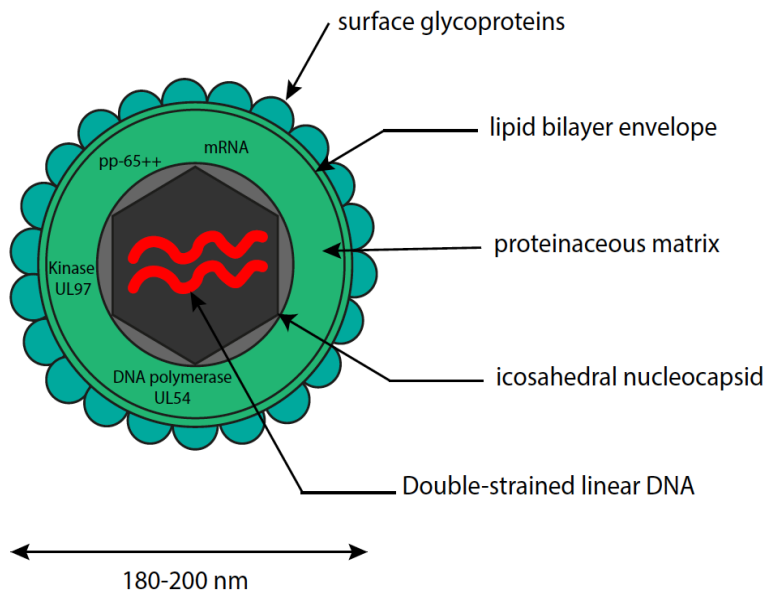
Figure 3 Structure of CMV particle

ii) Viral dissemination

100   Vertical and horizontal transmission of CMV have been described. Vertical transmission

101   occurs through transplacental and *intrapartum* transmission. Horizontal transmission is due to

102   organ transplantation from an infected organ or contact with infected body secretions. In a

103   first model, it is proposed that after an initial exposure, viremia associated with leukocytes

104   orchestrate a systemic diffusion in which organs such as the lung, spleen and liver are

105   infected. Finally, the virus undergoes a sequential dissemination in which the salivary glands,

106   breasts, and kidneys are infected (review (*12*)). Another model has shown how viral

107   transmission comes from upper respiratory tract (*12*). Alveolar macrophages and alveolar

108   epithelial cells of type 2 are first infected with endocytosis, that is facilitated by viral

109   glycoproteins gB, gH/gL/gO and by the pentameric complex gH/gL/UL128-UL130-UL131A.

110   The lytic cycle produces new infectious virus particles that infect other types of permissive

111   cells like fibroblasts, endothelial cells, dendritic cells and other innate immune cells,

112   including other alveolar macrophages. The dissemination occurs either through free-viral

113   particles infecting new cells or through cell-to-cell spread by direct cell-contact. Endothelial

114   cells play a crucial role in cell-to-cell spread and viral dissemination. They transmit the virus

115   by direct cell contact and during trans endothelial migration (*13*). Furthermore, infected

116   endothelial cells regulate adhesion molecules such as ICAM- and VCAM-1 which facilitate

117   the extravasation of infected monocytes and neutrophils and the transfer of the productive

118   virus to improve haematogenic spread (*14*). Virus infected cells may directly sow secondary

119   organs that lead to secretion into body fluids, where draining lymph nodes and blood filtration

120   organs are not a necessary stage prior to secondary organ infection (Figure 4).
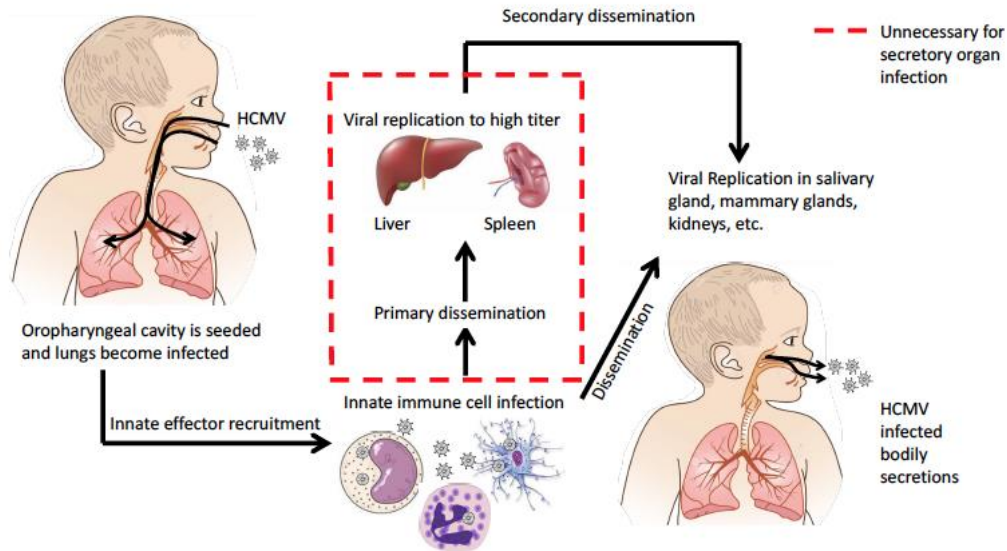
Figure 4 Alternative mechanisms of HCMV dissemination generated from animal model extrapolation (12).

In a first model, it is proposed that after an initial exposure, viremia associated with leukocytes orchestrate a systemic diffusion in which organs such as the lung, spleen and liver are infected. Finally, the virus undergoes a sequential dissemination in which the salivary glands, breasts, and kidneys are infected. Another model has shown how viral transmission comes from upper respiratory tract and could through infected leucocytes disseminate to all organs notably the secretory organs without a first step in the liver/spleen (12).

iii) Viral tropism

CMV has a very large tropism because it can infect a lot of human cell type, such as monocytes/macrophages, endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, stromal cells, neuron cells, neutrophils and hepatocytes (*15*), thereby contributing to CMV's high capacity to cause tissue damage in multiple organs. Heparan sulfate proteoglycans function to tether the virus to the cell membrane, through complex gN/gM interaction (*16*). Although strictly required, heparan sulfate proteoglycans are not solely sufficient for HCMV infection (*15*). The question of whether EGFR was a (co) receptor with integrins capable of recognizing gB when CMV entered fibroblasts was debated (*17-19*). Indeed, EGFR signaling has been observed after CMV entry (*17, 18*) but the promotion of virus-cell fusion by EGFR and the internalization of virion components to the cytoplasm linked to EGFR were not demonstrated. The hypothesis was that integrins binding to gB participate in the activation of EGFR signaling, since both $\beta 1$ and $\beta 3$ integrins have been shown to associate with EGFR and

142   activate it in a ligand-independent manner (*20*). Viral entry of CD34+ cells has been

143   associated with EGFR-gB interaction with EGFR signaling (*21*) but similarly to fibroblast,

144   direct binding and internalization has not been shown and one of the major mechanisms

145   highlighted for CMV entry in CD34+ cells was micropinocytosis (*22, 23*). Conversely, EGFR

146   signaling has been involved in CMV entry into monocytes (*24*) and the direct binding of

147   EGFR independently of β1 integrin and its internalization has been recently demonstrated

148   (*25*). gH/gL/gO is required for the entry of CMV into endothelial/epithelial cells and

149   fibroblasts and PDGFRα was identified as its ligand (with direct interaction between

150   PDGFRα and gO) but is expressed only on fibroblasts (*26*), therefore the ligand(s) on

151   endothelial and epithelial cells remain(s) unknown. The pentameric gH/gL/UL128-131

152   complex has been involved in CMV entry in leukocytes, epithelial, endothelial (*27*) and

153   dendritic cells (*28*). Neuropilin-2 has recently been identified as a receptor of this pentameric

154   complex and its interaction have been shown to be necessary for the viral entry into epithelial

155   and endothelial cells (*29*) but the ligands of the pentameric complex host cell are not known

156   for the other cell types. Finally, our group has demonstrated that DC-SIGN binding gB also

157   contributes to the CMV entry in dendritic cells (*30*). The scheme below summarizes the type

158   of cells which may be infected and the viral-protein/host cell receptor known to be involved

159   in CMV entry (Figure 5).

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iv) Viral cycle

The viral cycle starts after infection of the host cell which may occur either by direct fusion or through the endocytic pathway. The virus binds to the cell through interactions between viral glycoprotein and specific surface receptors described earlier. After this step, the fusion of the envelope with the cell membrane occurs to release the nucleocapsids in the cytoplasm. These nucleocapsids transfer to the nucleus, where viral DNA is released. This initiates Early Immediate (IE)-1/ IE-2 gene expression. Viral replication and maturation are followed by the stimulation and parallel accumulation of viral synthesis function. This process involves the encapsulation of replicated viral DNA as capsids, which are then transported from the nucleus to the cytoplasm. Secondary envelopment occurs in the cytoplasm at the mid-compartment of the endoplasmic reticulum (ER)-Golgi. It is followed by a complex two-step final envelopment and egress process that leads to virion release by exocytosis at the plasma membrane (*9*). Three successive phases occur during CMV replications (Figure 6): very early (or IE for Immediate Early, 0 to 2 hours), early (E, early, <24 hours), and late (L, >24 hours), with each step controlling progress to the next. Schematically, IE proteins are responsible for

179 the positive and negative regulation of viral and cellular genes and control their own

180 expression. E proteins are critical for genome replication. The DNA is then replicated and

181 during the late phase, structural proteins are synthesized. The CMV replication cycle is slow;

182 in infected human fibroblasts, new virions are released after 48 to 72 hours. The maximum

183 quantity of virions is released within 72 to 96 hours. Viral production continues over several

184 days at a high level. The host cell eventually dies after 4 or 5 days (*31*).

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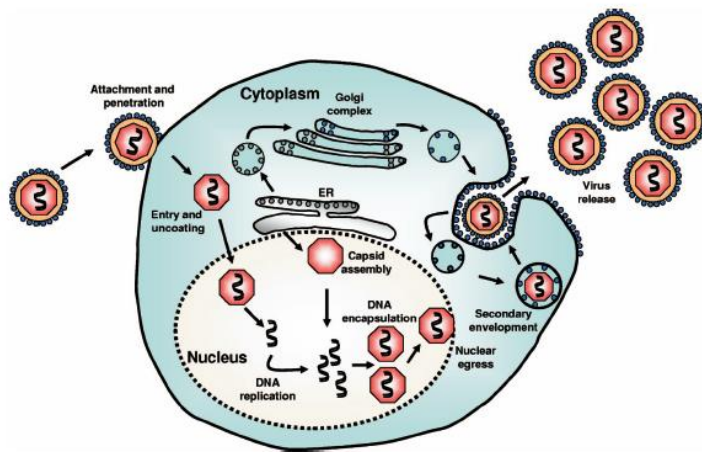
186 *Figure 6 Life cycle of HCMV in a human cell.*

187 *First, the virus enters into its host cell (direct fusion or endocytosis),the nucleocapsid from the cytoplasm is*
188 *translocated into the nucleus, where DNA is released. This initiates IE1-2 gene expression. Viral replication and*
189 *maturation involves the encapsulation of replicated viral DNA as capsids, which are then transported from the*
190 *nucleus to the cytoplasm. Secondary envelopment occurs in the cytoplasm at the endoplasmic reticulum (ER)-*
191 *Golgi intermediate compartment. This is followed by a complex two-stage final envelopment and egress process*
192 *that leads to virion release by exocytosis at the plasma membrane (9).*

193

III. Interaction between CMV and its human host

195 As mentioned earlier, CMV and its numerous hosts have evolved together for 80 millions of

196 years. CMV is only found in mammals, representing one of the classes with the most

197 advanced immune system (Figure 7). One of the specificity of CMV is its ability to use cell

198 host machinery to better replicate and encode components that help its viral escape and thus

199 promotes its host persistence. One can imagine that CMV replication and evasion qualities

200 have been acquired during its long-term co-evolution with the pressure selection of the virus

201 genes and with the integration of host genes into its own genome. This redesign of the viral

202 genome has led for example to the coding of "homologous viral-proteins" that are very

203 similar to host-proteins and are mostly involved in host evasion.

204

205
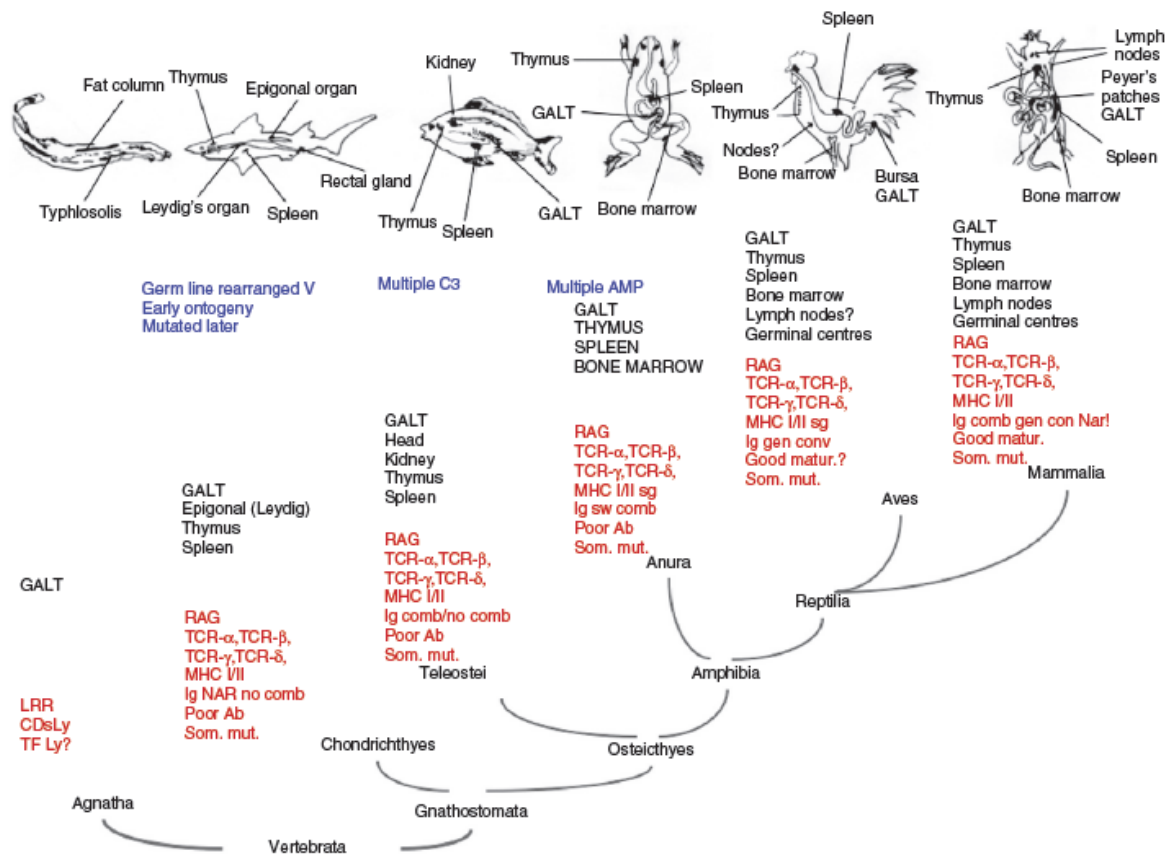
206 *Figure 7 The evolution of the immune system within vertebrates.*

207 *AMP, antimicrobial peptides; C3, third component of the complement; CD ly, CD determinants of lymphocytes;*
208 *GALT, gut-associated lymphoid tissue; LRR, leucine-rich repeat; MHC, major histocompatibility complex; Poor*
209 *Ab, antibody response with no or weak affinity maturation in opposition to good maturation (good matur.); Som.*
210 *mut., somatic mutation; RAG, recombination associated gene; TCR, T-cell receptor; TF Ly, transcription factor*
211 *specifying lymphocyte development. In blue are represented new mechanisms or acquired function in the*
212 *immune system in the corresponding specie (ie Germlin rearranged V appeared in the first vertebrates*
213 *(agnathans). In black are represented the organs and in red the components of the immune system of each specie*
214 *(32)*

A. CMV has optimized the persistence in its host

i) By optimizing its viral replication

HCMV encodes several proteins that contribute to maintain viral translation and replication in its host cell in order to prevent spontaneous cellular response to stress induced by its infection, normally resulting in decreased translation and cellular apoptosis (*33*). Indeed, human cytomegalovirus shares a general lifecycle strategy with other mammalian double-stranded DNA viruses that replicate within the nucleus to accommodate host cell: increased glucose uptake, metabolism and oxygen utilization; removal of cell growth controls (*33*). They manipulate the cell cycle to an optimal point for virus growth; and inhibit apoptosis during the productive phase of replication. These massive cell changes normally result in reticulum stress and decrease translation capabilities, including translation. HCMV has been shown to be able to maintain translation by modulating PI3K/AKT/mTOR signaling. HCMV activates AKT (phosphorylation of Threonine 308 on AKT by PI3K) which in turn results in the activation of mTORC1 with 4EBP and S6 phosphorylation. The virus also inhibits other mechanisms in the cell stress response that may negatively control mTORC1 activation (*33*), both of which contribute to cell survival during the viral replication phase. Moreover, reticulum endoplasmic stress could be regulated by the modulation of the unfolded protein response (UPR) (*34*), thereby contributing to the maintenance of protein translation. CMV may also act on the post-translational stage by interacting with the cellular proteasome. Indeed, CMV codes for proteins which catalyze the ubiquitylation of host proteins, thus promoting their degradation (*35*). It also may encode proteins that indirectly alter the conjugation of Ub or Ub-like proteins by altering the abundance and/or activity of cell ubiquitin ligases (*36*); or remove existing Ub fragments by their deubiquitinase activity (pUL48) (*37*). The biological relevance of deubiquitination for CMV replication was highlighted when observing the impact of a mutation of this enzymatic center that

241 compromises the nearly tenfold replication capacity (*37*). Incorrect localization of checkpoint

242 proteins by HCMV also contributes to inhibit the DNA damage response (*38*). These different

243 functions could influence the cell cycle, the regulation of the response to DNA damage, and

244 finally the prevention of cell death/apoptosis (for review(*39*)).

245
246   **ii) By persisting under latent form**

247 One original way that CMV persists is its ability to become latent. Latency is the presence of

248 viral DNA without protein expression. CMV establishes latency in haematopoietic progenitor

249 cells (HPC) CD34+ and monocytes CD14+ (*40*), while endothelial cells as candidates for

250 latency for HCMV remain elusive (*41*).

251 Latency mechanisms have not been well understood. However, this knowledge was enhanced

252 by the use of recombinant viruses containing substitutions or stop codon insertions to disrupt

253 a single open reading frame (sORF). This has led to know that the growth restriction has been

254 conferred to UL138 and the replication advantage to UL135 and studies are helping to

255 understand their antagonistic role. Both genes are present on the same UL133-UL138 gene

256 locus and are separated by UL136. The three genes have a different kinetic expression as

257 UL135 is expressed in the early phase of infection and UL136 and 138 in the early and late

258 phase of infection, which may favor latency at the late phase (Figure 8).

259 In latently infected primary CD34+ HCPs, the expression of UL138 was detected, but

260 conversely that of UL135 (*42*) was downregulated. UL138 is needed for virus latency in

261 CD34+ HPC because its disruption results in failure of entering in latency (*43*). UL138

262 repressive capacity is partly due to prevent demethylation of histones. This subsequently

263 causes the viral genome to be silenced and in particular prevents the activation of the

264 immediate early promoter (*44*). UL138 acts also by activating EGFR, which promotes

265 EGFR/PI3K/AKT signaling and finally suppresses viral replication (*45*). Moreover, UL138

266 induces EGR1 through MEK/ERK signaling. As a result, UL138 via EGR-1/EGFR (*46*)

267 stimulates its own gene expression (*47*) creating a positive equilibrium from its antagonistic

268 counterpart UL135. Furthermore, increased levels of EGR-1 contribute to the maintenance of

269 the CD34+ progenitor phenotype (*48*). On the other hand, UL135 reduces the total and cell

270 surface levels of EGFR (*47*), thus downregulates EGFR signaling. The MiR-US22 (small

271 regulatory RNA) targets EGR-1 and induces its top-down regulation which reduces the

272 expression of UL138 and thus promotes reactivation (*49*).

273 Finally, UL136 codes for 5 isoforms: UL136p23/p19 are pro-latency proteins, while

274 UL136p33/p26 promote virus replication and UL136p25 isoform could be implicated in

275 either case depending on the context (*50*).

276
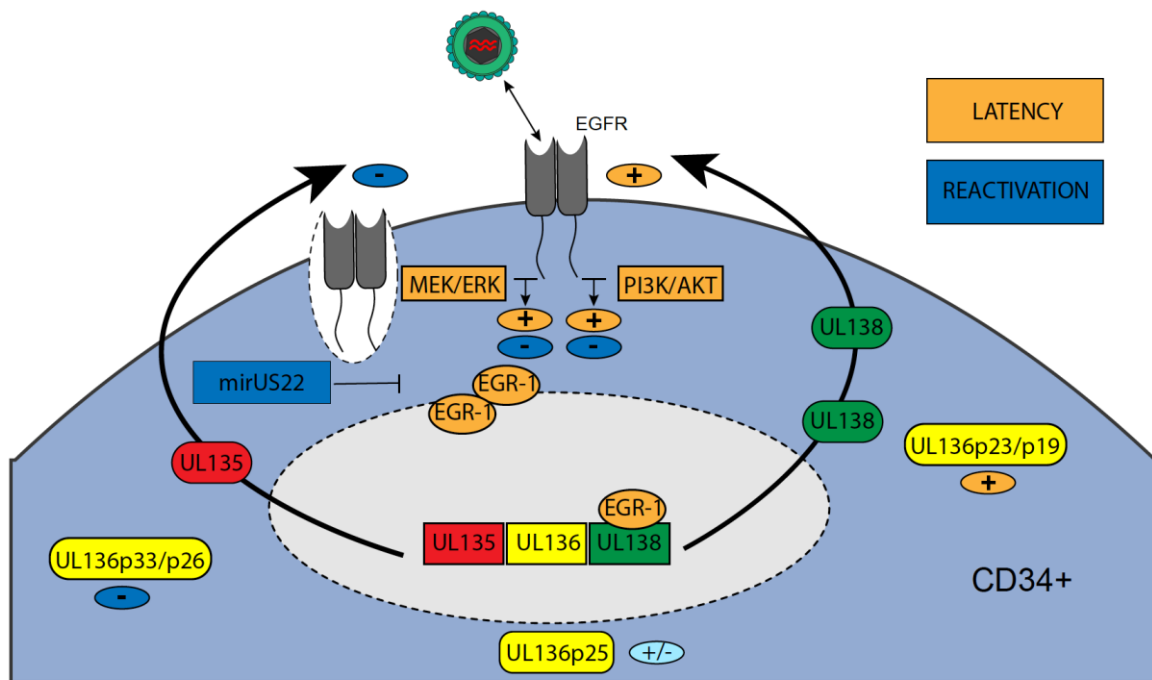
277 *Figure 8 Modeling of latency/reactivation balance mechanism.*

278 *UL138 and UL135 are located on the same UL133-UL138 gene locus, separated by UL136. After CMV entry in*
279 *human cell, the expression of UL138 increase EGFR signaling which in turn activate MEK/ERK and PI3K/AKT*
280 *patways. EGFR/PI3K/AKT signaling suppresses viral replication.. MEK/ERK induce EGR-1activation which*
281 *induces in turn UL138 transcription. UL135 downregulates EGFR membrane expression which decrease EGFR*
282 *signaling. UL136 could express isoforms either involved in latency or reactivation*

283

284   **B. CMV has optimized its evasion of the immune system**

285 CMV also optimized its persistence by evading the immune system. Here, we will review the

286 various players in the immune system, describe the mechanisms used by the CMV to escape

287   them and illustrate it in Figure 9. As discussed above, CMV infects species (mammals) that

288   have acquired the different types of immune actors and thus co-evolved together until now

289   (Figure 7).

290   Long-term cohabit could have promoted recombination with cellular DNA or post-integration

291   of cDNA derived from spliced cellular mRNA (*51*), which confer abilities for CMV to encode

292   homologous molecules able to interact with almost every immune actor.

293   We will see that studies have shown the mechanisms for evading CMV to counter all players

294   of the immune arsenal and we will discuss which mechanisms could alter γδ T cell response,

295   for which only hypotheses can be made.

296

297   **i)   IFN I/PRR/inflammasome pathway**

298   Pattern recognition receptors (PRR) act as the first line of defense because they can detect

299   pathogens at the cell surface or just after pathogen infection of their host cells, especially in

300   epithelial cells, in monocytes and dendritic cells. The PRR studies have demonstrated how

301   CMV can be recognized on the surface of cells, in cytosol, and in endosomes. The Toll like

302   receptors (TLR) are the best characterized PRR and recognize hydrophobic molecules in the

303   plasma membrane and nucleic acid in the endosomes and the cytoplasm. TLR2 recognizes gH

304   and gB in monocytes and fibroblasts (*52, 53*) but the exact phenomenon leading to its

305   downstream signaling is not fully understood. TLR2 activation leads to NF-κB activation,

306   type I IFN response by monocytes (*54*) and expression of pro-inflammatory cytokines such as

307   IL6 and TNFα (*55*). In mice, TLR9 recognizes CMV DNA in endosomes via the downstream

308   adaptor molecule myeloid differentiation factor 88 (MyD88). MyD88 activates IRF7 (*56*) and

309   induces the production of inflammatory cytokines, including IFN type I by plasmacytoid

310   dendritic cells (pDC) or classic DC (cDC) with a first peak 8 hours post-infection. In humans,

311   TLR9 is only expressed in B cells and pDC. This pDC TLR9/MyD88/IRF7 axis contributes to

312   activate NK cells (*57, 58*) and CD8+ αβ T cells, involved in CMV clearance (*56, 58*). Finally,

313   DNA sensors such as DAI and IFI16 could recognized CMV DNA independently of TLR and

314   also lead to type I IFN production, which can explain why IFN I could be produced

315   dependently of MyD88 but independently of TLR (*59*). Cytosolic DNA could also be

316   detected by AIM2, involved in the activation of inflammasomes which results in caspase-

317   dependent IL1β/IL18 maturation in human and mouse macrophages (*60, 61*). In MCMV-

318   infected macrophages, this sensor has a critical role in IL1β and IL18 production. Production

319   of IL-18 was demonstrated to be dependent on AIM2 during *in vivo* MCMV and directly

320   affects the production of interferon-gamma (IFNγ) by natural killer cells within 36 hours of

321   infection (*62*). Finally, the IFI16 sensor is also capable of binding CMV DNA in human

322   fibroblasts and induced anti-viral cytokines such as IFNβ (*63*).

323   CMV has developed a counterattack strategy that blocks these innate functions. pUL83 (pp-

324   65) was identified for direct binding to IFI16 resulting in reduced cytokine production after

325   activation of the IFI16 pathway (*63*). pUL83 also binds AIM2 reducing similarly

326   inflammasomes-dependent AIM2 activation (*64*). IE86 inhibits the binding of NFκB to the

327   IFN-β promoter, inhibits TNFα-induced NFκB DNA binding (*65*) and also blocks NFκB-

328   dependent transcription of pro-ILβ in macrophages (*66*). In addition to inflammatory signals

329   after TLR/DNA sensing, dendritic cells also play a role during CMV infection in producing

330   IL12 (which positively improves NK cell (*67*) and γδ T cell response (*68*)) and in activating

331   the adaptive part of CMV immune responses (*69, 70*). UL111a codes for two IL-10 homologs

332   that modulate DC functions. It represses the maturation of monocytes to dendritic cells by

333   downregulating the expression of class II HLA, by decreasing co-stimulatory receptors such

334   as CD80, CD83, CD86, and CD40 (*71, 72*), CD11c expression, and by reducing the

335   acquisition of dendritic phenotype. UL111a also decreases TNFα, IL6, IL1β gene

336   transcription and TGFβ, TNFα and IL6 protein production by CD34+ cells (*72, 73*), and

337 promotes DC apoptosis as well (*71, 74*). Moreover, UL111a contributes to inhibit type I

338 interferon (IFNβ/α) production by pDC (*75*). Lastly, UL111a induces a M2 polarization of

339 monocytes with the downregulation of CD14, CD163 and class II HLA expression which

340 limits their CD4+T cell priming, and also inhibits their HO-I (Heme oxygenase) activity

341 which is involved in TNFα and IL1β production (*76*).

342

343   **ii) Immunoglobulins**

344 Following B cell maturation, immunoglobulin G (IgG) are produced mainly against gB (*9,*

345 *77*) and less often against gH (*78*). Their role in controlling primary CMV infection is minor

346 since B-cell depleted mice can recover (*79*). However, anti-CMV IgG play a role in the

347 control of CMV reactivation since B-cell depleted mice are subjected to higher viral

348 load/dissemination during a CMV reactivation induced by immunodepression (*79*). The

349 transfer of immune serum demonstrates that the lack of antibodies allowed the virus spread in

350 deficient mice during reactivation (*79*). Anti-CMV IgG start to rise 7 days after the onset of

351 infection and reach neutralizing titers after two weeks (*79*). Anti-CMV IgG and IgM lead to

352 free virion opsonization/neutralization and target viral antigens on infected cells, with lysis

353 capacity of target cells in the presence of complement (*80, 81*). These IgG could also bind

354 cellular receptors for the Fc domain of IgG (FcγR) on the cell surface of NK cells and Vδ2[neg]

355 γδ T cells which leads to antibody-dependent cell-mediated cytotoxicity (ADCC) (*82*) or

356 antibody-mediated cell inhibition by the production of IFNγ (*68*). UL118 and TRL11 genes of

357 HCMV have been identified as encoding gp68 and gp34 respectively as FcγR counterparts

358 inhibiting IgG binding to NK cells (*83*).

359

iii) NK cells:

NK cells expand within few hours after CMV infection begins (*62*), with an initial type I IFN and IL12 priming (*84*). They are involved in the disease recovery in mice (*84, 85*) and act on target-infected cells by direct cytotoxicity and by activation and recruitment of other cells of the immune system through their cytokines and chemokines secretion, including IFNγ and TNFα (*84*). A specific phenotype of NKG2C^hiCD57+ was described in the CMV response and showed abilities to create a pool of memory NK cells (*86-89*). Their activation mode is supported by cumulative signals from inhibitory and stimulatory receptors. Inhibitory receptors with inhibitor tyrosine-based inhibitory motifs (ITIM) such as killer cell-Ig-like receptors (KIRs), leukocyte Ig-like receptor 1 LILRB1 (LIR-1 also called CD85j), and C-type lectin receptor CD94/NKG2A transmit a negative signal by recognizing class I HLA or class I HLA homologs, such as MICA/MICB and HLA E. As will be further elaborated, HCMV lowers the cell surface HLA molecule expression that would lead to decrease inhibitory signals for NK cells. Interestingly, HCMV UL18 gene encodes a class I HLA homolog that binds peptide and β2microglobulin and recognizes LIR-1/CD85j (*90*). Structural analyses showed an increase in the contacts of UL18/peptide/LIR-1 complex and an optimal surface complementarity in the LIR-1/UL18 interface, compared to the LIR/class I HLA interfaces, resulting in a >1,000-fold higher affinity (*90*) and thus restoring LIR-1 inhibitory function. LIR-1 is expressed on other cells of the immune system, including dendritic cells (*91*) and CD8 αβ T cells (*92*). For example, the binding of pUL18 to DC impairs cell migration and CD40 ligand-induced maturation (*93*).

HLA-E is recognized by the inhibitory CD94/NKG2A dimer when it binds a peptide derived from class I HLA molecules and by CD94/NKG2C, an activating receptor, when it binds other kind of peptides (*94*). A nonameric peptide derived from pUL40 sequence (a class I HLA homolog) is a ligand for HLA-E and promotes HLA-E expression on the cell surface,

385   facilitating interaction between HLA-E and CD94/NKG2A, and thus leading to inhibition of

386   NK cells (*95*).

387   On the other side, HCMV downregulates the ligands of stimulatory receptors to escape to NK

388   cell lysis. The ligands of NKG2D are the human major histocompatibility complex (MHC)

389   class I chain-related genes (MIC) A, MICB, and ULBP1-6 (UL Binding Protein) molecules,

390   which are particularly expressed during viral stress (*96*). pUL16 prevents cell surface

391   expression of both MICB and ULBP1-2. MICA and ULBP-3 expression are prevented by

392   pUL142 and UL122 which specifically blocks MICB (*97*) by sequestration into the

393   endoplasmic reticulum or Golgi (*98*). pUL141 blocks the adhesion molecules CD155

394   intracellularly (*99*), thereby preventing its recognition by DNAM-1 (*100*). Finally, pp65

395   dissociates the ζ-chain from NKp30 thus blocking its signaling (*101*).

396   Interactions between NK cells and CMV genes are the perfect example of host-virus co-

397   evolution. Most viral proteins leading to the escape of NK cells are host-counterparts

398   suggesting that the replication of CMV in its host cells has borrowed genetic material that in

399   turn helps to encode viral counterparts, especially HLA or HLA-like molecules. The host has

400   selected counterattack strategies as well. For example, MICA*008 does not bind pUL142, so

401   it may persist on the surface of host membrane and be recognized by NKG2D (*102*).

402   Moreover, MICA and MICB downregulation are mediated by viral proteins produced during

403   intermediate early phase of CMV infection (*103*). As NK cells may be activated at an early

404   stage, this open a short "window" for NK cell control. Studying these mechanisms of host-

405   virus interactions allowed the identification of new host proteins important during immune

406   response, as it is the case for LIR1 (discovered during studies on UL18) and ULBP proteins

407   (discovered during studies on UL16).

408

iv) αβ T lymphocytes

αβ T cells comprise CD4+ and CD8+ T cells. Their crucial role in controlling CMV has been illustrated by clinical situations in which they are affected such as in HIV-induced acquired immune deficiency syndrome or during immunosuppressive treatments, blocking activation signals of T cells. Our understanding on the specific role of each subset is derived from mouse models, which differentiate the role of CD4+ and CD8+ αβ T cells from NK cells in cell immunity. Polic *et al.* used sequential and additional depletion of CD4+, CD8+ αβ T cells and NK cells. They showed their partial redundant role with 5.6% of CMV recurrence with NK depletion alone, 11.7% with CD4+ T cell depletion alone, 4% with CD8+ αβ T cell depletion alone, 24% with NK and CD4+ T cell depletion, 80% with NK and CD8+ αβ T cell depletion, 100% with the depletion of the three subsets in B-cell depleted mice (*85*). αβ T cells recognize peptides either presented by class II HLA molecules on antigen-presenting cell (CD4+ T cells) or presented by class I HLA molecules expressed on target cells (CD8+ αβ T cells). The frequency of open reading frame (ORF) recognition by CD4+ T cells showed that peptides from UL55 (gB), pp65, UL86 and UL99 (pp28) were the most commonly recognized (*104*). Those from UL48, pp65, IE1 and IE2 were the most frequently identified by CD8+ αβ T cells. In peripheral blood, following CMV infection, CD4+ T cells (*105*) start to rise 7 days later, while CD8+ αβ T cells start to increase 20 days after CMV infection begins (*106*). Kinetics of anti-CMV IgG, CD8+ and CD4+ αβ T cells were compared in symptomatic and asymptomatic transplant patients during CMV primary infection. It reveals that anti-CMV IgG and CD8+ αβ T cells increase similarly in both situations (*106*) while CD4+ T cell expansion was delayed only in symptomatic patients, which shows the relevance of CD4+ T cells in the control of CMV in immunocompromised hosts. Moreover, CMV-specific CD4+ T cells appear to have a specific function as compared to CD4+ T cell during other antigen encounters. During primary CMV infection, a specific subset of CD4+CD28-

434   granzyme B+Perforin+ T cells expands, and is found only in CMV+ individuals (*107*). They

435   can produce TNFα, MIP-1β during CMV-peptides stimulation (*107*) and may lyse target cells

436   expressing CMV antigens in a class II-dependent manner (*108*). These observations suggest

437   that this specific effector memory CD4+ T cell subset is particularly important to control

438   CMV infection, compared to conventional IL2-producing memory CD4+ T cells that

439   remained in the lymph nodes (*109*). However, the lysis has not been consistently observed,

440   which suggests a CMV evasion effect even if antigen presentation remains (*110*).

441   Cytotoxic CD8+ αβ T cells have also shown their protective effect, notably in mouse model

442   with CMV-specific CD8 T cells transfer (*111*). They produce massive amounts of IFNγ and

443   have cytotoxic activity on CMV-infected target cells (*106*). During the primary phase of

444   CMV infection, a phenotypic change is observed since they become CD27+, CD28+,

445   CD45RO+, CD45RA-, CCR7-, Perforin+, Granzyme B+, Ki67++ (*106*). Lately, in the

446   chronic phase of CMV infection, memory CMV-specific CD8+ αβ T cells are separated into

447   two subsets: The central memory CD27+CD28-CD45RA+, with proliferation capacities after

448   TCR restimulation (also seen in other chronic viral infections) and the "terminally

449   differentiated effector memory cells", also called TEMRA, CD27-CD28-CD45RA+ with low

450   proliferation capacity, but high cytotoxicity that appears to be highly present specifically

451   during CMV infection (*112*).

452   Even strongly efficient during CMV infection, CD4+ and CD8+ αβ T cells could be

453   counterattacked by a CMV escape mechanism, targeting class II and class I HLA expression.

454   In the early phase of CMV replication, the peptide derived from IE1 transcription factor

455   presented in a class I HLA molecule is one of the first antigenic signal for CD8+ αβ T cell

456   cytotoxicity (*113*). This could be blocked by pp65 phosphorylation of IE-1, preventing an IE-

457   1-specific CTL response (*114*).

458   In addition US2, US3, US6, US10, and US11 block the generation and/or the export of HLA

459   class I/peptide complexes and induce rapid downregulation in class I HLA expression (for

460   review, (*9*)). US3 retains class I HLA into the endoplasmic reticulum (ER); US2 and US11

461   mediate cytosolic degradation of heavy chains by the proteasome (*115*), notably because

462   US11 in the ER membrane dislocates the new class I HLA from ER to cytosol (*116*). US6

463   interacts with transporters associated with antigen processing (TAP) in the endoplasmic

464   reticulum and interferes with the transport of class I molecules due to defective peptide

465   translocation (*115*). US10 delays the normal trafficking of class I HLA out of the ER (*117*).

466   US3 binds β2-microglobulin-associated heavy chains that are thus retained in the ER (*118*).

467   US2 also induces class II downregulation by promoting proteasome degradation, especially

468   HLA-DRα and DMα (*119*).

9A
viral evasins

hours   days   weeks   years
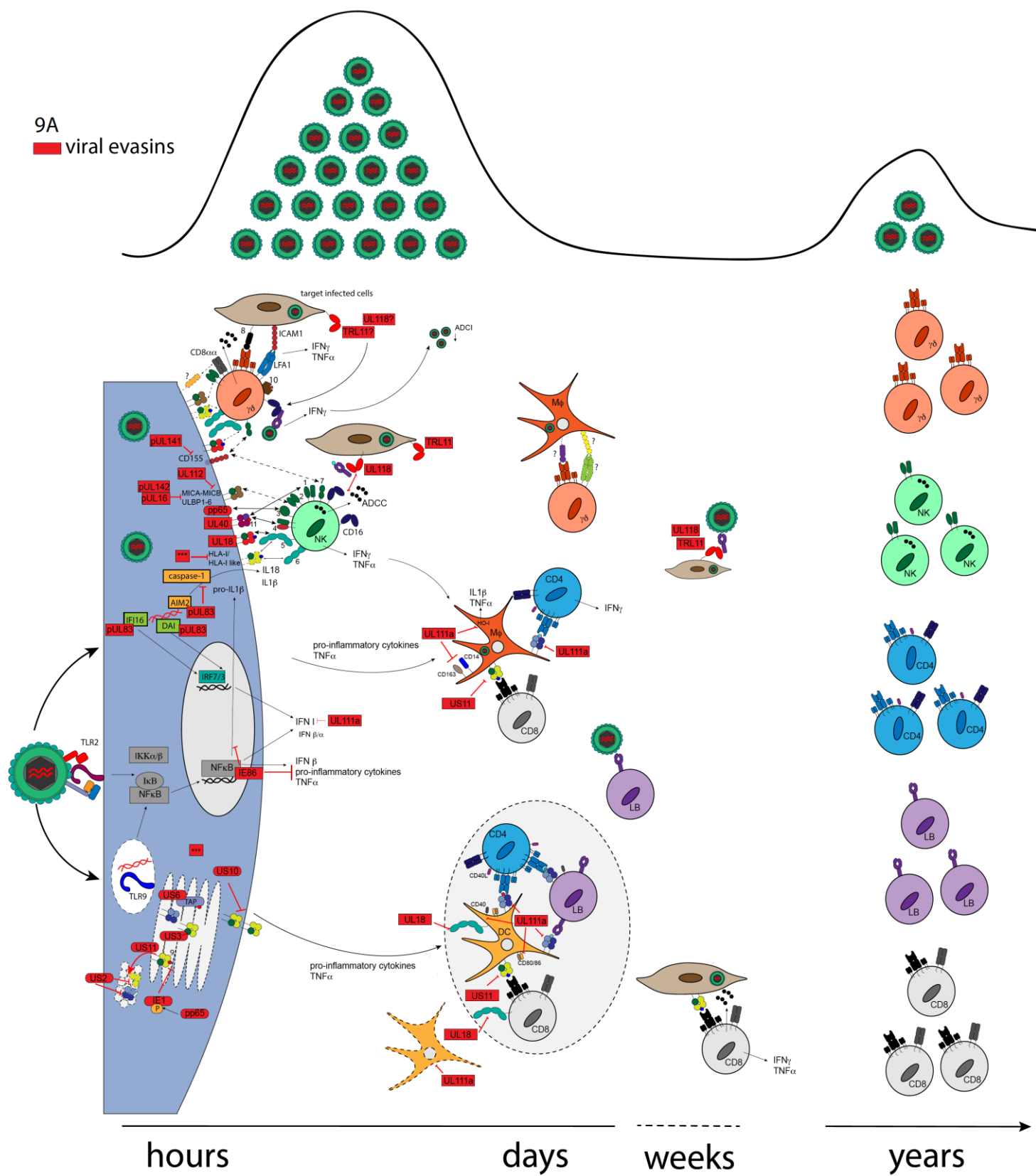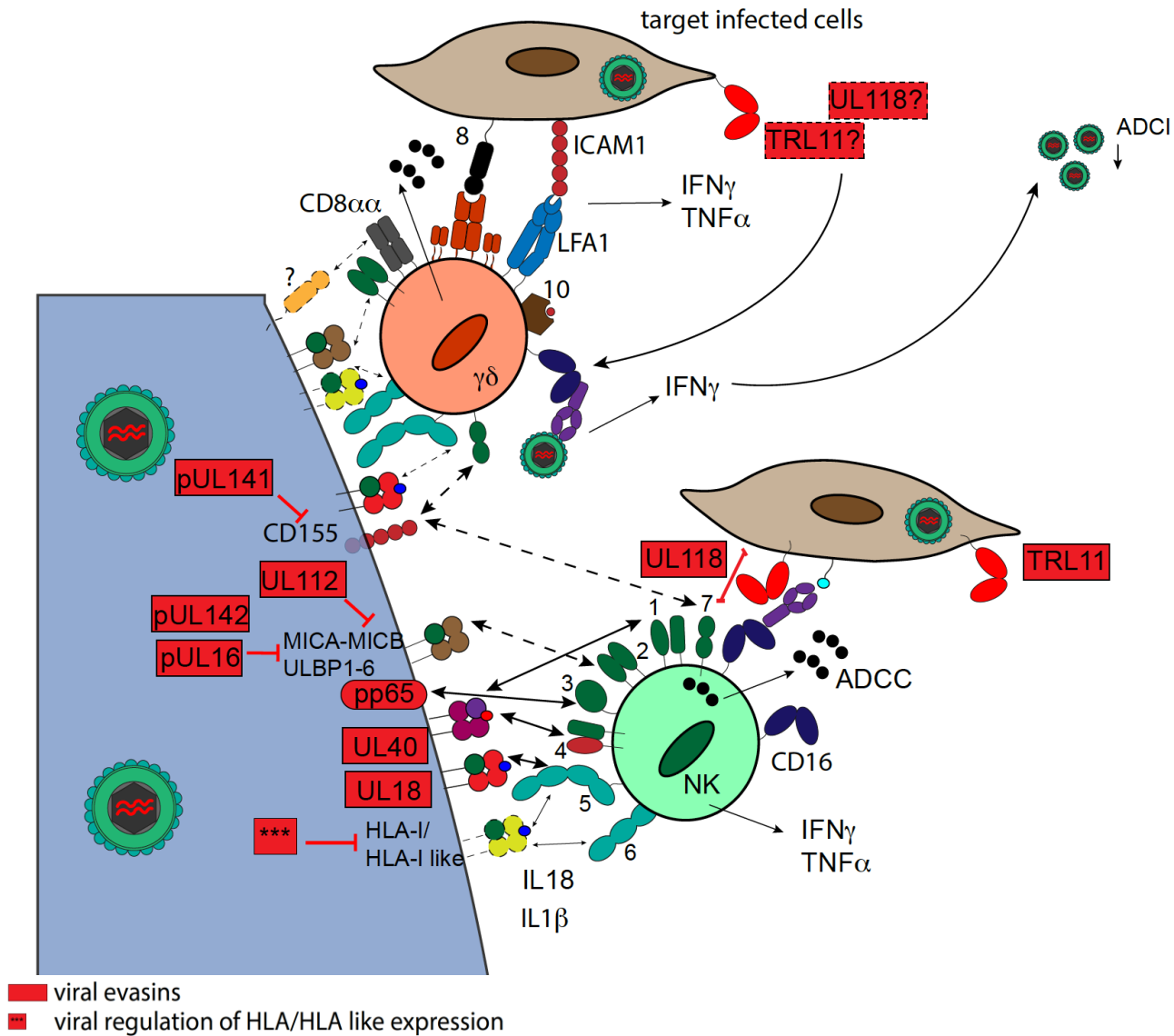
Figure 9 Interplay between CMV immune response and viral evasion

9A. Full model of immune system/CMV interactions. 9B. Focus on γδ and NK cell interactions. ADCC, antibody-dependent cell cytotoxicity; ADCI, antibody-dependent cell inhibition; DC, dendritic cell; MΦ, macrophage; IFI16, interferon inducible protein 16; DNA-dependent activator of interferon; MICA/B, MHC class 1 polypeptide–related sequence A/B; ULBP, UL binding proteins; IL, interleukin; TLR, toll like receptor; IFN, interferon; TNF, tumor necrosis factor. 1. NKG2C 2. NKG2D 3. NKp30 4. CD94/NKG2A 5. LIR-1 6. KIR 7. DNAM-1 8.EPCR, Annexin A2, unknown 9. IE1 peptide 10. IL18-receptor. 11. HLA-E.

480 **v) γδ T lymphocytes**

481 The last identified player in cell-mediated immunity against CMV was the γδ T lymphocytes

482 (*120, 121*). We have reviewed here their original development, their mode of recognition and

483 functions, their particularities regarding the evasion of CMV, and integrate them in the

484 specific immune response to CMV.

485 Although these criteria have been challenged, we are still attempting to classify the immune

486 response as "innate" or "adaptive". "Innate" define immune actors that are present at the

487 antigen site of entry, that can get activated very quickly after antigen recognition but are

488 poorly specific with highly conserved and weakly diversified antigen receptors. Conversely,

489 the "adaptive" response is a term dedicated to actors who are not initially in the tissues, which

490 need a priming to activate, in this way more slowly but which express a specific antigen

491 receptor and develop long-term memory, providing long-term antigen-specific protection.

492 This memory fate has been well studied for B cells, αβ T cells and more recently for NK cells

493 in the context of CMV, although they do not express a specific antigen receptor.

494 As they express a specific T cell receptor for antigen, γδ T cells have therefore been

495 frequently described by comparison to B cells or conventional αβ T cells. Like conventional

496 αβ T cells, γδ T cells differentiate in the thymus where they acquire TCR expression, Vγ and

497 Vδ chains, but TCR recognition is independent of the molecular histocompatibility complex

498 conversely to αβ T cells. All these receptors present a common ability for "theoretical"

499 specificity and diversity obtained by somatic gene rearrangements from a limited number of

500 V (Variable), D (Diversity), J (Junction) and C (Constant) genes and by junctional diversity

501 with addition or deletion of nucleotides between the different gene segments when they are

502 joined together, also called somatic recombination. This diversity is mainly generated in the

503 complementary-determining region 3 (CDR3) of the T-cell antigen receptor (TCR) or B-cell

504 antigen receptor (*122*) that allows a broad-spectrum generation of antigen-specific receptors.

505 However, in humans, the TCR chains type and their diversity, their pre-acquired function, and

506 their location are used to distinguish Vγ9 Vδ2 γδ T cells from the others collectively called

507 non-Vγ9 Vδ2 γδ T cells, which are those involved in CMV response.

508 The Vγ9^{pos}Vδ2^{pos} γδ T cells are thought to be innate-like cells and are the predominant subset

509 in the peripheral blood. The studies on their development give arguments for defining them as

510 an innate subset. Dimova *et al.* found that during fetal life, Vγ9Vδ2 γδ T cells exhibited a

511 restricted Vγ9Vδ2 TCR diversity, including for more than 50% of them a germline-encoded

512 sequence, with pre-programmed effector function (IFNγ, granzyme A-K). They develop in a

513 sterile environment in the thymus, and represent the predominant γδ T cell subset circulating

514 in human fetal blood (*123*). The TCR diversity repertoire of Vγ9Vδ2 TCR is amplified with

515 increased N-additions in postnatal individuals even though it is still restricted in the thymus,

516 and is not related to fetal exposure to microbial antigens, suggesting a second wave of thymic

517 production after birth (*124*). Conversely, the Vγ9Vδ2 TCR repertoire within adult blood is

518 generated from the postnatal thymic Vγ9Vδ2 TCR repertoire with further expansion into the

519 periphery following post-birth microbial exposure (*124*) (Figure 10).

520

528   Their predominant presence in the peripheral blood does not contradict their innate ability to

529   activate directly at the site of infection, given the example of *Plasmodium falciparum*

530   surveillance which is usually a blood infection (*125, 126*). Their limited TCR diversity relates

531   to their restricted type of activation. Vγ9Vδ2 γδ T cells recognize small phosphorylated

532   intermediate metabolites of the isoprenoid synthesis pathways, also called phosphoantigens

533   (pAg), such as isoprenyl pyrophosphate (IPP) and (*E*)-4-Hydroxy-3-methyl-but-2-enyl

534   pyrophosphate (HMBPP) (*127*). Sensing of these cellular phosphoantigens by Vγ9Vδ2 γδ T

535   cells is mediated by interactions between members of the butyrophilin family and the Vγ9Vδ2

536   TCR. Butyrophilins (BTN) are part of the immunoglobulin superfamily. They are

537   glycoproteins consisting of two extracellular immunoglobulin domains, stabilized with

538   disulfide bonds: constant IgC, and variable IgV and a transmembrane region.

539 In humans, the family of butyrophilins includes 7 butyrophilin proteins, 5 butyrophilin-like

540 proteins and the SKINT-like factor (*128*). We know since 2014 that the intracellular B30.2

541 domain of the BTN3A1 binds pAg (*129*). However the binding of the germline-encoded HV4

542 region of the Vγ9 chain (*130*) by BTN2A1 has been discovered more recently as essential for

543 the TCR-dependent activation by pAg (*131*). These observations suggest an additional CDR3-

544 dependent signal transmitted by pAg that has not yet been identified.

545 The other subset, the CMV-responding non-Vγ9Vδ2 cells, is preferably localized in epithelial

546 tissues. It has been more described as an adaptive subset because of the diversity of their TCR

547 repertoire, its delayed expansion kinetics common to those of CD8+ αβ T cells and its

548 acquisition of memory phenotype (*132, 133*). However, recently, the development of human

549 γδ T cell has shown two different waves of non-Vγ9Vδ2 γδ T cell production, leading to

550 innate-like and adaptive-like cells (*134*). Indeed, during fetal life only, haematopoeitic stem

551 cell precursors coming from the liver are responsible of a first wave of invariant non-Vγ9Vδ2

552 γδ T cell production, including the generation of effector invariant germline-encoded Vδ1Vγ8

553 T cell that were functionally programed (IFNγ and Granzyme B) (*134*) and show CMV

554 reactivity. The invariance of the TCR was linked to a higher expression of Lin28b that

555 inhibits the terminal deoxynucleotidyl-transferase (TDT) responsible for the random addition

556 of nucleotides during VDJ recombination, thus reducing TCR diversity. After birth and

557 adulthood, there is a higher expression of TDT in the thymus, the production γδ T cells comes

558 from haematopoetic bone marrow precursors and leads to polyclonal cells without pre-

559 established functions (*134*) (Figure 11) with naive phenotype (*135*). A continuum between

560 invariant and polyclonal non-Vγ9Vδ2 T cells is assumed because naive polyclonal non-

561 Vγ9Vδ2 T cells have also been observed during fetal life, also expanding during CMV

562 infection (*136*).

563   As a result, both invariant innate-like and polyclonal non-Vγ9Vδ2 T cells can be produced

564   during early in life and are responsive to CMV.

565
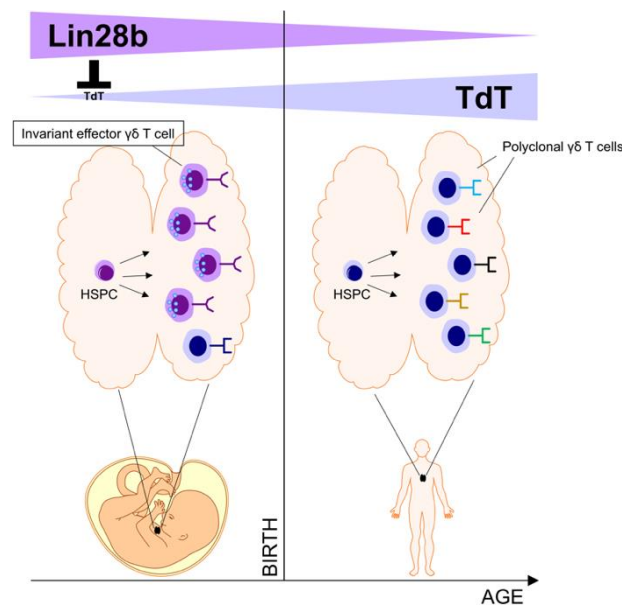
566   *Figure 11 Non-Vγ9Vδ2 waves production during fetal life and post-natal period.*

567   *Graphical abstract of Paola Tieppo et al. J Exp Med 2019;217(3). Non-Vγ9Vδ2 wave production during fetal*
568   *life includes germ-line encoded sequences, then during post-natal period with, non-Vγ9Vδ2 TCR sequences*
569   *present a highest diversity involving N-addition through high terminal deoxynucleotidyl-transferase (TdT)*
570   *expression.*

571

572   In addition, a third counterpart of γδ T cells expressing Vδ2 chain without Vγ9 chain was

573   identified during fetal life and presented CMV-mediated expansion (*136*), but it was thought

574   to disappear after birth (*123*).

575   In adult, CMV-reactive γδ T cells were mostly described as adaptive cells. We recently

576   reviewed their characteristics during CMV, regarding their expansion, their phenotype, their

577   recognition mechanisms, and their capacity to evolve to memory cells (**ARTICLE 1**) (*137*).

578   We recall in particular that during CMV infection, they make a transition from the naive stage

579   to the TEMRA differentiation similarly as conventional αβ T cells (*132, 133*), they present a

580   clonal expansion suggesting an antigen-driven recognition (*137*), and they are able to expand

581   faster during reinfection (*138*). However, unlike the Vγ9Vδ2 counterpart, it is still difficult at

582   this time to generalize their mode of recognition, even in the most studied infectious context

583   in which they are activated, including CMV, because a very low number of antigenic ligands

584   for TCR has been identified. Moreover, in the context of CMV, two mechanistic models of

585   recognition are opposed. We identified Annexin A2 as a "self-stress" antigen, which is

586   upregulated at the surface of the cell membrane during CMV infection and lead to the direct

587   Vγ8Vδ3 clone activation. Its "stress-induced" nature was explored through other kind of

588   stress such as cell confluence, oxygen deprivation, thermic shock and tumoral transformation,

589   all leading to both Vγ8Vδ3 JRT3 and clone activation. TCR recognition was both

590   demonstrated by direct interaction and specific TCR signaling (**ARTICLE 2**) (*139*). By

591   contrast, our group also identified EPCR which is expressed in the opposite way

592   constitutively at the cell surface of CMV-infected cells. Vγ4Vδ1 clone TCR recognizes EPCR

593   but requires the co-signaling of LFA-1/ICAM-1, and ICAM-1 was the ligand upregulated at

594   the membrane surface of CMV-infected cells (*140*).

595   Even though no link has been made with CMV so far, it is important to note that TCRs

596   expressed by non-Vγ9Vδ2 T cells have also been shown to bind and to be activated by

597   members of the butyrophilin family. By contrast to the recognition of antigens mainly by the

598   CDR3 region of the TCR, binding to butyrophilins relies on germline-encoded motifs within

599   the cognate TCR variable γ-chains qualifying this recognition as innate-like.

600   This recognition of butyrophilin could also assign a tissue-specific localization of some

601   populations of γδ T cells for rapid activation, an innate-like characteristic. In mice, the tissue-

602   specific assignation and activation signal given by Btnl1(*141*) or butyrophilin-like "SKINT-

603   1"(*142*) has been observed for the semi-invariant Vγ7+ T cells in enterocytes and for the

604   invariant Vγ5 T cells in keratinocytes respectively. In humans, BTNL3+8 have been shown to

605   activate Vγ4 intraepithelial γδ T cells (*143*). Finally, BTN2A1 showed a binding on the

606   germline encoded region of the Vγ9 chain of a Vδ2Vγ9 TCR but have also shown to bind Vγ9

607   chain of non-Vδ2Vγ9 TCR (*130*). Consequently, non-Vδ2Vγ9 γδ T cells can present an

608   innate-like activation through a TCR invariant region recognition of butyrophilin family

609   member and an adaptive activation through the TCR CDR3 recognition of specific antigen.

610   In the context of CMV in adulthood, the interaction between butyrophilins by TCR-chains has

611   not been elucidated and requires further investigations. Their tissue localization and response

612   kinetics during CMV, contributing to the assessment of their innate properties, are difficult to

613   evaluate in humans and knowledge of these features come from mice.

614   Indeed, during the course of CMV infection in humans, kinetics of expansion parallel that of

615   αβ T cells but their preferential tissue location suggests earlier intervention. For this purpose,

616   mouse models provided more information (*144*). Although they are not necessary to control

617   infection, γδ T cells protect CD3ε KO and Rag$^{-/-}$γc$^{-/-}$ mice from CMV-induced death (*144*).

618   In this model, γδ T cells were found in tissues on day 3 of CMV infection (not tested at earlier

619   times-points (*144*)). They were a less common IFNγ producer than NK cells, but they were

620   able to produce IFNγ also from day 3. These observations contribute to ascribing innate-like

621   characteristics to γδ T cells during CMV infection, in contrast to the diversity of their TCR

622   (*145*). Therefore, the innate-like properties presupposed by CMV-responding γδ T cells

623   combined with their well-described adaptive features with the establishment of long-term

624   memory. In addition, the γδ T cell recognition mode against CMV involves receptors-ligand

625   in which CMV evasins have not been identified (for Annexin A2, EPCR or ICAM-1).

626   However, they also expressed DNAM-1, CD85j or KIRs after CMV infection, which can be

627   counterattacked by CMV evasins, thus the role of these receptors for reactivity of γδ T cells

628   against CMV has to be evaluated. For CD16, theoretical inhibition by FcγR CMV homolog

629   could also exist and has to be demonstrated. They can thus present original activation method

630   which are not targeted by CMV proteins, innate like potential for rapid activation on the site

631 of infection and long-term memory. These characteristics prompt us to focus on them in order

632 to improve our knowledge about these original players of the immune response to CMV.

633

634 **C. Improving our understanding of γδ T cell role in the immune response to**

635 **CMV**

636 We therefore continued to explore the "repertoire" of γδ T cell responding to CMV, analyzing

637 the ability of the third $V\gamma9^{neg}V\delta2^{pos}$ subgroup to specifically respond to CMV in

638 immunocompromised adults. They have first been considered as a third subset of γδ T cells

639 since they express the Vδ2 chain independently of the Vγ9. This subtype has been mostly

640 described in early life (*136*) but was thought to involute after birth. Conversely, these

641 $V\gamma9^{neg}V\delta2^{pos}$ T cells were recently found to persist in adulthood, and similarities regarding

642 their phenotype and their TCR repertoire diversity compared to those of $V\delta2^{neg}$ subgroup

643 have been also demonstrated (*146*). We found that these $V\gamma9^{neg}V\delta2^{pos}$ T cells are also

644 components of the immune response to CMV, while exhibiting distinct characteristics of the

645 $V\delta2^{neg}$ γδ T cells. CMV sero-positivity was the single clinical parameter associated with the

646 expansion and differentiation of the $V\gamma9^{neg}V\delta2^{pos}$ T cells. Longitudinal analysis demonstrated

647 substantial cytotoxic potential and activation during acute CMV primary infection or

648 reinfection. Their reactivity to CMV was tested *in vitro,* showing the relative specificity

649 without activation against other herpesviruses and a T cell receptor-dependent production of

650 IFNγ against CMV-infected cells. Their unique role compared to $V\delta2^{neg}$ γδ T cells was

651 observed regarding the clinical characteristics of CMV infection, since $V\gamma9^{neg}V\delta2^{pos}$ T cells

652 and not $V\delta2^{neg}$γδ T cells subset exhibited an expansion correlated with CMV severity.

653 Therefore, this work identified a new player in the immune response against CMV in

654 immunocompromised adults and opened interesting clinical perspectives for using

655 $V\gamma9^{neg}V\delta2^{pos}$ T cells as an immune biomarker for CMV disease severity (**ARTICLE 3**) (*147*).

It also improves our understanding of the human nomenclature of γδ T cells, which may definitively separate from a functional perspective the Vγ9Vδ2 γδ T cells from all others.

We also deciphered the signature of γδ T cells among PBMC and cancer tissues through the first single-cell RNA analysis of purified γδ T cells. This work helped us to distinguish what may define γδ T cells, notably from CD8+ αβ T cells and NK cells. This also allowed to separate the Vγ9Vδ2 signature from that of the CMV-induced Vδ2^{neg} γδ T cells. Thirty-eight genes were differentially expressed between the two populations. These comprised 25 genes, such as KLRC2 (NKG2C), KLRF1 (NKp80), FCGR3A (CD16), GZMB, and GZMH, which were more expressed in TCRVδ1 cells than in TCRVδ2 cells, and 13 genes, including KLRC1 (NKG2A), GZMK, LTB, and IL7R, which were up-regulated in TCR Vγ9Vδ2 cells. Vδ1 γδ T cells signature of CMV+ donors was also compared to the one of CMV- donor: Vδ1 γδ T cells from CMV+ donors express highly NKG7, GZMH, GZMM, FCGR3A, FGFBP2, PRF1 compared to those from CMV- donor. Conversely, Vδ1 γδ T cells from CMV- donor express more frequently CCR7, CD27 IL7R (signature of naive cells). This work also demonstrated the technical feasibility to detect γδ T cells using a single-cell RNA signature, with the potential for reproducible studies in peripheral blood or tissues (*148*) (**ARTICLE 4**).

Except in the context of early life, the repertoire of naive γδ T cells in humans has been poorly investigated. This can be explained by the quasi absence of naive Vγ9Vδ2 T cells in adults and by the late differentiated status of non-Vγ9Vδ2 γδ T cells in CMV-seropositive adults (that represent about half of the population in western countries). We thus took advantage of the naive status of non- Vγ9Vδ2 γδ T cells in CMV-seronegative adults to investigate their repertoire and functions. We addressed this issue in terms of their quantitative and qualitative response to CMV, exploring the existence of a natural repertoire of γδ T cells responding to CMV.

680 We thus began to study the repertoire able to recognize CMV-infected cells in individuals

681 naive for CMV. We observed that the phenotype of naive $V\delta2^{neg}$ γδ T cells had close

682 similarities with naive αβ T cells but, in contrast to naive αβ T cells, they exhibited rapid and

683 specific reactivity against CMV, with or without the addition of IL18 (a cytokine able to

684 increase the TCR-dependent production of IFNγ) (*149*)). The amount of these CMV-

685 responsive γδ T cells comprised an average of 0.25% of IFNγ producers; 0.58% to 2.2% of

686 CD69/CD71 double-positive cells without or with IL18 respectively. A single-cell analysis

687 was performed from sorted naive $V\delta2^{neg}$ γδ T challenged or not with CMV. Unbiased UMAP

688 analysis deciphered a cell cluster consisting solely of $V\delta2^{neg}$ γδ T cells that had been

689 challenge with CMV and accounted for 35% of that condition. This observation suggested

690 that those 35% of naïve $V\delta2^{neg}$ γδ T cells present common gene expressions that have been

691 regulated during CMV challenge and are different from the gene expression of all other naïve

692 $V\delta2^{neg}$ γδ T cells. Moreover, they also could represent the largest observable proportion of

693 naive CMV-responding Vδ2neg γδ T cells. We analyzed the differential gene expression

694 characterizing this cluster. First analysis revealed the predominant gene expression of

695 IL18RAP (coding for IL18 receptor accessory protein), CFS2 (coding for GM-CSF), CD160,

696 CCR1 (receptor of CCL3/MIP-1a, CCL5/RANTES), CCL7 and CCL23, IFNγ and TNFSF9

697 (CD137). Conversely, CCR7 was one of the most downregulated, suggesting that naive

698 $V\delta2^{neg}$ γδ T cell that respond to CMV in 24h *in vitro* have already evolved toward a

699 maturation process compared to others. These findings are encouraging to support the

700 existence of a natural repertoire of CMV-responding $V\delta2^{neg}$ γδ T cells among individuals who

701 have never been in contact with CMV (**DRAFT 1**).

702

IV. Focus on CMV interaction with its human immunocompromised host: the example of kidney transplantation

703

704

705 The interaction between CMV and its human host is complex with a long co-evolution that

706 have led to the development to many CMV mechanisms to escape the host's immune

707 response. In immunocompetent individuals, the vast majority of cases are asymptomatic and

708 the virus persists without clinical manifestations. During primary infection, this well-

709 controlled replication is explained by the rapid establishment of an immune response. (Figure

710 12A). Then, asymptomatic viral replication alternates with latent phase, leading to strong and

711 inflationary immune response of memory cells (Figure 12B). In immunocompromised hosts,

712 such kidney transplant recipients, CMV replication may lead to clinical symptoms, sometimes

713 severe. Two situations can be observed in clinical practice. In the first case, the infection

714 occurs in an immunosuppressed patient before he or she has been able to establish an immune

715 response against CMV. This consists in a primary infection and constitutes the more life-

716 threatening situation. Immunosuppression delays the establishment of the immune response,

717 the patient is symptomatic and requires an antiviral drug until efficient immune response

718 against CMV is achieved (Figure 12C). In the second case, CMV may reactivate or

719 superinfect while the patient is already immunized against CMV. In this situation, patients are

720 less likely to be symptomatic than the patients with primary infection, probably because their

721 pre-established immune response against CMV is sufficient to control the virus (Figure 12 D).

722 However, in some cases, CMV replication could be symptomatic and the immunological

723 reason why remains elusive. This prompted us to evaluate what is the fate of the immune

724 response against CMV in this clinical scenario (Figure 12 E).

A. primary infection in an immuncompetent host
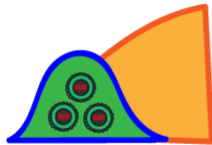
B. new infection episod in an immuncompetent host
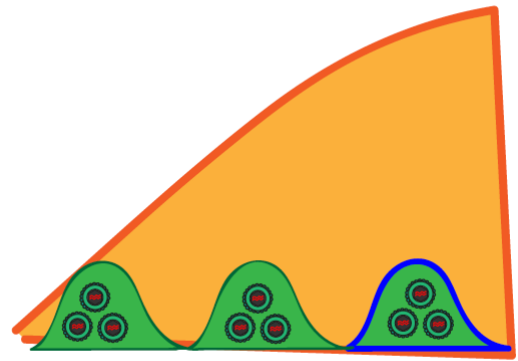
C. primary infection in an immuncompromised host

antiviral drug
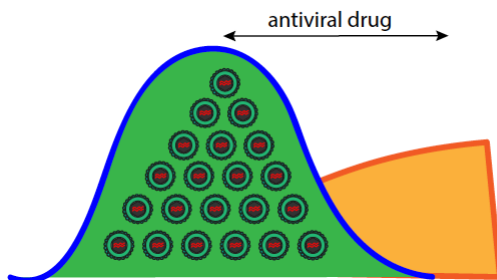
D. new infection episod in an immuncompromised host
First scenario
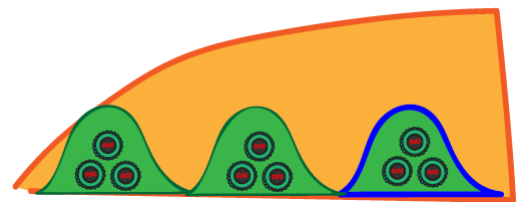
E. new infection episod in a immuncompromised host
Second scenario

antiviral drug

immune response

previous viral episods

viral episod of interest
for the current scenario

725
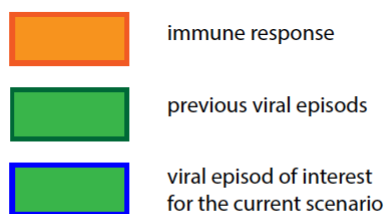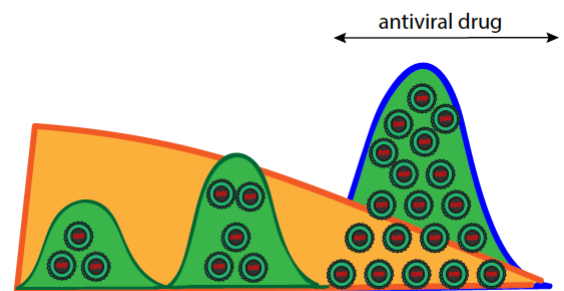
Figure 12 Propositions for viral load and immune response kinetics.

In case of primary infection in an immunocompetent host (A), in case of a new episode of replication (reactivation) in an immunocompetent host (B), in case of primary infection in an immunocompromised host (C), in case of a new episode of replication (superinfection or reactivation) in an immunocompromised host with hypothesis to understand why in this situation replication be either well-controlled (D) or uncontrolled with clinical symptoms (E). The level of immune response can be seen as quantitative but also qualitative/functional state in its ability to prevent invasive CMV replication.

A. Clinical aspects of CMV disease in kidney transplant patients

CMV infects a large part of the population at a very young age. Seroprevalence ranges from 30% to 90% worldwide. In Western Europe, it is around 50% at age 50 (*9, 150, 151*). In solid organ transplantation, CMV can be responsible for: (i) primary infection in CMV-negative patients receiving an organ from a CMV-positive donor (D+R-), (ii) reactivation in CMV-positive patients receiving an organ from a CMV-negative donor (R+/D-) or (iii) reactivation and/or superinfection in CMV-positive patients receiving an organ from a CMV-positive donor (D+R+). In the context of organ transplantation, the virus is associated with significant morbidity and sometimes with higher mortality (*152*).

Infection has been previously defined by detecting viral pp65 by immunofluorescence using specific monoclonal antibodies in the nucleus of circular polynuclear cells and now by detecting viral DNA by molecular techniques (CMV PCR) , a more sensitive technique (*153*). CMV disease comprises two entities: (i) CMV viral syndrome, which includes an infection associated with fever, malaise, leukopenia, thrombocytopenia or hepatic cytolysis, and (ii) invasive CMV disease, which is defined by organ damage associated with the detection of the virus in this organ by molecular biology or immunohistochemistry techniques. In some cases, organ-specific damage associated with virus detection in the blood leads to the diagnosis of CMV disease without evidence of tissue virus detection (such as CMV colitis) (*153*). A broad spectrum of organs could be affected by CMV infection such as retinitis, brain, colon, liver, pancreas or lungs (*153*). Moreover, CMV infection has been associated with indirect effects in solid organ transplants, such as T-cell mediated rejection (*154, 155*) but whether this is a cause or a consequence remains unclear. Indeed, if CMV occurs before acute rejection, the risk of rejection could be increased because of the clinical decision to decrease immunosuppressive treatment as prevention of CMV disease, or *vice versa* if acute rejection occurs before, CMV infection could be favored by increased immunosuppression to treat

758   rejection. In heart transplantation, it has been associated with an increased cardiovascular risk

759   with cardiac allograft arteriosclerosis (reviewed in (*156*)). CMV has also been associated with

760   a poorer graft survival (*157*) and increased mortality (*158*). Issues relies on improving the

761   prevention of CMV infection and the curative treatment to avoid CMV persistence,

762   recurrence and emergence of mutant strain.

763

764   **i) CMV prevention**

765   Over the past 20 years, the advent of highly sensitive CMV PCR and the development of

766   effective oral antiviral therapies, such as valaciclovir and valganciclovir led to two preventive

767   strategies for patient management, thereby redefining the epidemiology of CMV in organ

768   transplantation. First, universal prophylaxis involves a preventive antiviral treatment for 3

769   months (R+ patients) to 6 months (D+R- patients) post-transplantation (*152, 159*). Second, a

770   pre-emptive strategy can be proposed based on a close monitoring of infection through blood

771   PCR CMV weekly screening in the first months after transplantation, and the initiation of the

772   antiviral treatment only if an infection occurs (*152, 159*). The incidence of infection and

773   disease in the population at risk is therefore different depending on the strategy chosen and, of

774   course, depending on the donor and recipient CMV serology at the time of transplantation. In

775   the absence of preventive treatment, CMV infection occurs in 60-80% *(160)* and CMV

776   disease in 50% (*155*) in the D+R- subgroup of patients, and 20% in the R+ subgroup (*161*).

777   Universal prophylaxis or preemptive strategy reduces the incidence of infection/disease. In

778   D+R- patients, CMV DNAemia occurs in about 37% with a 6-month prophylaxis (*162*),

779   between 34% (*163*) to 75% (*163*) with a 3-month-prophylaxis and between 54% (*164*) and

780   83% (*164*) with a preemptive strategy. The incidence of CMV disease among D+R- patients

781   varies between 16% (*162*) to 34% (*165*) with a 6-month prophylaxis, between 16% (*162*) to

782   36% (*162*) with a 3-month prophylaxis and between 8% (*166*) to 33% (*164*) (mean 20%) with

783 a preemptive strategy (Table 1). In R+ patients, CMV DNAemia occurs between 11% (*167*)

784 to 38% (*167*) with 3-month prophylaxis and between 37% (*167*) to 78% (*167*) with a

785 preemptive strategy. CMV disease occurs between 4% (*167*) to 8% (*167*) with 3-month

786 prophylaxis and 7% (*167*) to 13% (*167*) with a preemptive strategy (Table 2). With respect to

787 the quite equivalent proportions of CMV with a preemptive or prophylactic strategy, both are

788 recommended in the current guidelines. However, each strategy has *pros* and *cons* that may

789 help the clinical center to choose between both of them. Preemptive strategy does not protect

790 from CMV infection, which may be associated with more indirect effects (*155, 164*), is more

791 difficult to implement and does not prevent other herpesviruses. Universal prophylaxis has the

792 disadvantages of late-onset disease and adverse drug effects (150). The best preventive

793 strategy option may be implemented through immune surveillance as recently reviewed (*137*)

794 (**ARTICLE 1**) and interventional studies using immune-monitoring are ongoing. Indeed,

795 individualized preventive strategy could be performed combining preventive strategy and

796 immunomonitoring:

797   - No prevention for patients with a strong cellular mediated immune-response before

798 transplantation who will not receive lymphodepleting agents (*168, 169*),

799   - preventive strategy until cell-mediated immunity is detected.

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CMV DNAemia (%)	D+R-				n
	Prophylaxis	preemptive	mTORi	Ongoing mTORi	
Walker, 2007					171
McGillicuddy, 2010		37			130
Weclaviack, 2010	28	67			282
Witzke, 2011	11	38			296
Couzi, 2012	38	78			96
De Sandes-Freitas, 2018		66.7	60 (PCR/2w)		19
Tedesco-Silva, 2015		83*	55.5* (Ag-pp65/w)		13
Cristelli, 2018		78	74		89

808 *Table 1 Studies about preventive strategies in D+R- patients.*
809 *Universal prophylaxis is compared to preemptive strategy and preemptive strategy is compared alone or with*
810 *everolimus based immunosuppressive regimen. *Difference between groups was not statistically significant; w,*
811 *week; Ag-pp65, antigenemia pp65*

CMV DNAemia (%)	R+				n
	Prophylaxis	preemptive	mTORi	Ongoing mTORi	
Walker, 2007					171
McGillicuddy, 2010		37			130
Witzke, 2011	11	38			296
Couzi, 2012	38	78			96
De Sandes-Freitas, 2018		52.2	10.4 (PCR/2w)		98
Tedesco-Silva, 2015		57.4	11.7 (Ag-pp65/w)		242
Ferreira, 2019		71.6	13.6 (Ag-pp65/w)		169$
EVERCMV**		78	39 (PCR/w)	21.4	186
CMV disease %	R+				n
	Prophylaxis	preemptive	mTORi	Ongoing mTORi	
Walker, 2007	3	15			171
McGillicuddy, 2010	6	12			130
Witzke, 2011	4	13			296
Couzi, 2012	8	7			96
Ferreira, 2019		34.5	7.9		169$
EVERCMV**		11	4.5	***	186

812 *Table 2 Studies about preventive strategies in R+ patients*

813 *Universal prophylaxis is compared to preemptive strategy and preemptive strategy is compared alone or with*
814 *everolimus based immunosuppressive regimen. $ 91.7% of R+ patients; ** unpublished data;*** ongoing*
815 *analysis; w, week; Ag-pp65, antigenemia pp-65*

816

817 **ii) Curative treatment**

818 Finally, major issues about CMV infection/disease consist in the management of viral

819 persistence after a first treated episode, viral and clinical recurrences and the emergence of a

820 mutant strain (6% of CMV disease). Viral persistence and clinical recurrences have recently

821 been identified as the two major factors associated with the emergence of a mutant strain.

822 These situations seem to be clinical scenarios in which the antiviral drug exposure induce

823 selection pressure on a replicating virus leading to mutant strain emergence, by contrast with

824 the antiviral drug given during antiviral prophylaxis (**DRAFT 2**). Such entities (persistence,

825 recurrence, mutation) are actually the most difficult-to-treat CMV events, because of the

826 following non-exhaustive issues that remain unresolved:

827 - What is the appropriate duration of curative antiviral therapy with the risk of

828 emergence of mutant strains (*170*)?

829 - Could adjusting the duration of treatment based on immune surveillance be useful

830 (*137*)?

831 - How to manage immunosuppression during antiviral therapy (*152*)?

832 In conclusion, despite significant advances in molecular diagnosis and preventive strategies,

833 CMV remains the "troll of transplantation" (*171*). It is still the first opportunistic infection in

834 kidney transplant recipients (unpublished results, Figure 13) and further efforts are required

835 for a better control, in terms of reduction of incidence, curative treatment and prevention of

836 the emergence of mutant strains.

837
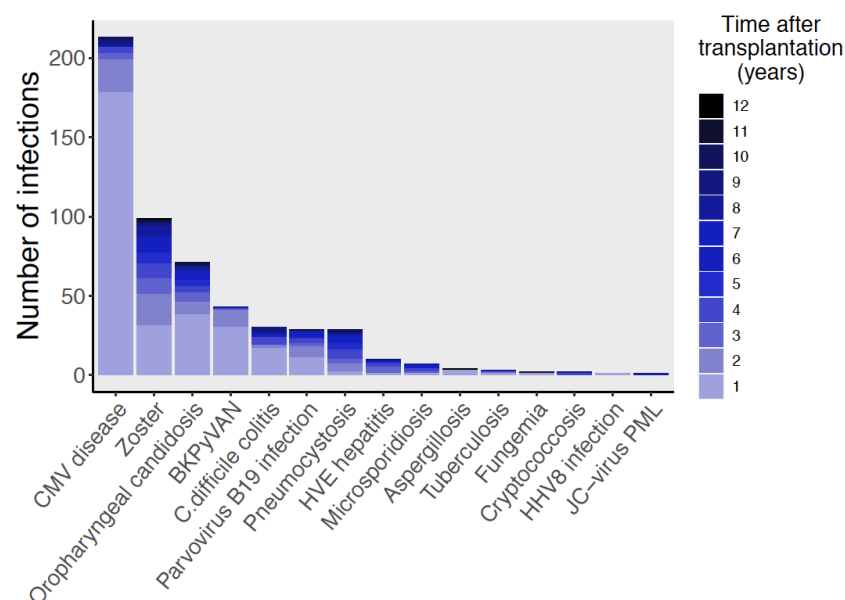
838 *Figure 13 CMV among opportunistic infections in kidney transplantation.*

839 *In Bordeaux University Transplant Center, patients transplanted for the first time between 2004 and 2015 were*
840 *included and were followed for at least two years. Clinical events associated with microbiological evidences*
841 *were included and were presented as number of events per year after transplantation. (personal unpublished*
842 *data, from a study performed with Drs Pierre Pfirmann and Benjamin Taton).*

B. Long-term kinetics of CMV immune response: an inflationary evolution

When CMV serology is well correlated with cell-mediated immunity, patients are assumed to exhibit functional immunity to control the virus (*172*). Indeed, inflationary memory is the classic fate of the immune response against CMV (*173, 174*). The term "memory inflation" is derived from studies using MHC Class I-peptide tetramers to study the CD8+ αβ T-cell response against MCMV (*175*). This allowed to describe a specific response in immunocompetent mice and to focus on a numerical expansion, which was observed at very late times, well beyond the initial control of infection (*173*).

It was suspected that CMV is a persistent chronic virus, undergoing repeated reactivations with low viral loads, presentation of renewed viral antigens, and thus inducing repeated CMV immune activation, leading to accumulation of CMV-specific T cells. This phenomenon has been illustrated by the fact that the T cell response to CMV in humans and mice is surprisingly large, comprising as much as 10%-20% of all CD8+ αβ T cells (*104, 175*). Subsequent criteria have been later assessed to complement the definition of "inflationary memory":

- Restricted contraction following priming, leading to a sustained long-term memory pool, which means the plateau is reached at a higher level than classic memory response (*176*)

- The phenotype of cells constituting the inflationary pool is generally an "effector memory" phenotype *i.e.* CD45RA+CD27-CD28-CCR7-CD62L- (*177*). In addition, "long-lived effector-type" phenotype has been distinguished for cells expressing the Hobit transcription factor and having high capacities to produce IFNγ and granzyme B and self-renewal (*178-181*).

- A sustained effector functionality. Indeed, despite their late-stage differentiation, they can clearly be driven to divide and appear to respond to viral reactivation by

869   expanding *in vivo* (*182*), killing target cells *ex vivo* and offering *in vivo* protection

870   (*182, 183*).

871   However, the maintenance of those functional effector T cells is probably more complex

872   than the long-term survival of the same terminally-differentiated cells. Indeed, these cells

873   also express KLRG1 which is rather present on short-term late differentiated cells (*184*).

874   Snyder *et al* showed that these highly functional T cells expressing terminal

875   differentiation constitute the «inflationary pool» and are also renewed regularly with the

876   recruitment of naive cells at a later stage in addition to the offspring of cells initiated

877   during the initial phase of infection (*174*). Overall, R+ patients are thought to have this

878   kind of CMV memory, which should give them long-term protection (Figure 14A).

879   However, other chronic and persistent viruses, such as LCMV clone 13, conversely result

880   in an "exhausted" long-term T cell response. This response is described when a high virus

881   load persists and induces a progressive hypo-responsiveness of antigen-specific effector T

882   cells (for review see (*185*)). These exhausted T cells present a dysfunctional state which is

883   characterized by an increased expression of inhibitory receptors such as PD-1, LAG3,

884   TIM3, a transcriptional program with high level of EOMES/low level of Tbet, and a

885   diminished capacity to produce cytokines and to proliferate (*186*) (Figure 14B). We will

886   see later that in the context of CMV, for some R+ patients, we also observed this

887   dysfunctional profile of T cells.

A. Viral kinetics associated with inflationary immune response

B. Viral kinetics associated with exhausted immune response

■ immune response

■ viral kinetics

888
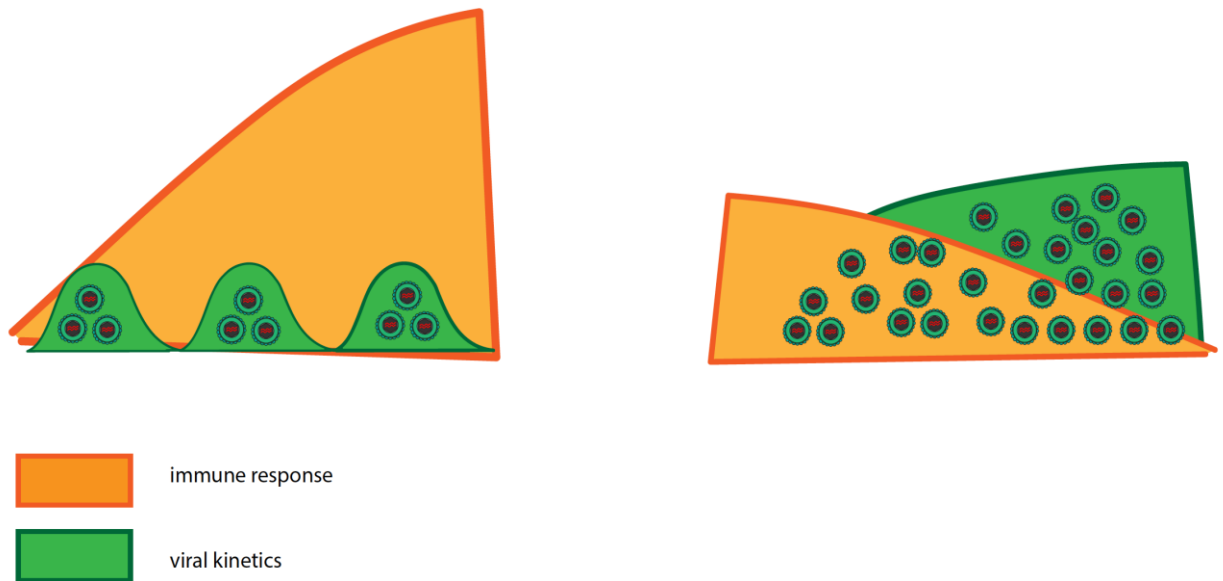
Figure 14 Viral kinetics supposed to induce inflationary (A) or exhausted (B) T cell response.

In 14A, intermittent low-viral load separated by latent phase has been described as inducing inflationary immune response. In 14B, high and prolonged viral load which has been described as inducing exhausted immune response

C. Improving our management of CMV preventive strategy in CMV seropositive patients.

One of the key issues in the CMV field of organ transplantation is the personalization of CMV prevention. Notably, in R+ patients, with strong "inflationary" T cell response, avoidance of prophylaxis or even preemptive strategy could be considered in combination with immune surveillance of T cell response at the beginning of transplantation. Results from Bestard's team are encouraging in this respect, showing that R+ patients with positive IE1-ELISPOT at day 15 of transplantation did not undergo CMV infection (*169*). In this context, the choice of the type of immunosuppressive regimen also offers new opportunities for CMV prevention.

After transplantation, T cell acute rejection is usually prevented by the instauration of two complementary drugs that target T cell activation and proliferation. After TCR activation, intracellular calcium influx activates phosphatases resulting in migration of NFAT

907 transcription factor into the nucleus. NFAT together with NFκB and AP-1 promote gene

908 transcription of IL2 and α chain of the IL2 receptor (CD25). The CD28/CD80/CD86 co-

909 stimulatory signal is needed to achieve full activation of the TCR signaling, including G1-

910 phase T cell engagement. Then, IL2 signaling induces mTOR activation leading to the

911 engagement in S phase with DNA replication and purine and pyrimidine bases synthesis that

912 are maximum during G2 phase. Immunosuppressive drugs target TCR-dependent signal

913 through inhibition of calcineurins (tacrolimus or ciclosporin), costimulatory signals (for

914 instance CTLA4-Ig, belatacept), CD25 (for instance anti-CD25 monoclonal antibody,

915 basiliximab), mTOR signaling (for instance mTOR inhibitors such as rapamycin/sirolimus or

916 everolimus), and purine base synthesis (for instance azathioprin or mycophenolic acid

917 (MPA)). Usually, immunosuppressive maintenance therapy combines a drug targeting

918 calcineurin with an anti-proliferative drug either mTOR inhibitors (mTORi) or mycophenolic

919 acid, but new drug combinations, notably with belatacept may be used to avoid nephrotoxicity

920 of calcineurin inhibitors (Figure 15).

Figure 15 Signaling pathways of T cell activation and action of the different molecules used as immunosuppressive drugs in transplantation.

Tac, tacrolimus; CsA, ciclosporin; MPA, mycophenolic acid; Aza, azathioprine

Recently, mTORi have been associated to a decreased CMV incidence, an observation that encouraged us to better understand this phenomenon through both a well-designed clinical trial and a mechanistic analysis of mTORi effects on CMV-specific T cell response.

i) mTOR signaling regarding the T cell differentiation status

931

932 The mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that has a

933 central role in the regulation of cell growth and metabolism (*187*). mTOR exists in two

934 multiprotein complexes in metazoans:

935 - mTOR complex 1 (mTORC1) contains a scaffolding protein, regulatory associated

936 protein of mTOR (RAPTOR), is sensitive to rapamycin, and receives upstream signals

937 from PI3Kinase and AKT. mTORC1 signaling causes the activation of S6 kinase

938 activation, which phosphorylates ribosomal protein S6 promoting ribosome

939 production. mTORC1 signaling also drives 4EBP1 to release eIF4E, involved in

940 mRNA translation. S6 phosphorylation leads to the negative feedback of AKT PI3K

941 phosphorylation at threonine 308 (T308) site (*188*).

942 - mTORC2 has a distinct scaffolding protein, is associated with a rapamycin-insensitive

943 companion of mTOR (RICTOR), and is relatively resistant to rapamycin, except under

944 prolonged periods of treatment (*188*).

945 Since mTORi currently used in the field of kidney transplantation mainly target mTORC1, we

946 will further develop their functions in T cells.

947 As described above, the most well-known mTOR role in T cells was to promote cell cycle

948 progression, but more recently, it has been studied in the regulation of immune receptor

949 signaling pathways, metabolism programs and migration (*189*). According to the type of T

950 cells, mTOR activation has a different role and the example of its effect on CD4+ T cell

951 differentiation has been the most studied. Under usual activation, rapamycin promotes CD4+

952 T cell differentiation into FOXP3+ regulatory T cells, it selectively leads to their expansion

953 (*190*) by improving metabolism and migration properties, whereas it inhibits Th1, Th2 and

954 Th17 T cells (*191*). Conversely, the specific deletion of mTORC1 (Raptor) in Treg cells leads

to loss of suppressive activity and fatal inflammation *in vivo* (*192*), suggesting that a basal

level of mTOR activity is required by Treg cells to maintain their function.

To better understand the role mTORi on CMV T cells response, we focused on the role of

mTORC1 in CD8+ αβ T cells, which impacts both immune signaling and metabolism.

Immune signals and insulin lead to mTORC1 activation. TCR and co-stimulatory signaling

induce rapid and intense mTOR activation whose magnitude is correlated with prolonged

TCR/CD28 engagement (*193*). IL12 enhances and prolongs TCR-dependent mTOR

activation, indirectly through STAT4 signaling, to program functional maturation of antigen-

stimulated CD8+ αβ T cells (*194*). TLR2 signal through MyD88 also participates in the

activation of mTOR and promotes the expression of Tbet (transcription factor favoring IFNγ

production) in a recently antigen-activated CD8+ αβ T cells. Finally, insulin may also

activate mTORC1 signal which promotes Tbet expression in a recent antigen-activated CD8+

αβ T cells (*194*).

The mTOR pathway activity also showed differential effects regarding the degree of CD8+

αβ T cell differentiation. In newly-activated cells, mTOR promotes effector functions

(cytokine production and cytotoxicity) (*195*) and contributes to the generation of T cell

memory (*196*). Naive cells are first quiescent and present a catabolic metabolism by which

they generate ATP via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation.

When activated, they markedly increase the uptake and consumption of glucose and

glutamine, with concomitant suppression of fatty acid oxidation (*197*). Upon TCR activation

in naive CD8+ αβ T cells, glycolysis and glutaminolysis participate in the synthesis of

biosynthetic precursors such as polyamines, which contribute to the T cell growth and

proliferation. The metabolic pathway between the glutaminolysis and the biosynthesis

polyamines has been shown to depend on MYC (*198*). Moreover, CD28 stimulation activates

PI3K/AKT axis, which increases glucose flux and glycolysis (*3*). A crosstalk between

980   PI3K/AKT and MYC through mTOR is suspected as in recently antigen-activated CD8+ αβ

981   T cells, rapamycin inhibits MYC expression and glycolysis induction while MYC-deficient T

982   cells fail to fully activate mTORC1 after TCR stimulation (*198*). The PI3K/AKT/mTOR

983   pathway activation via TCR and TLR2 promotes the synthesis of the Tbet protein, which in

984   turn results in increased binding to IFNG, GZMB and PRF1 gene promoters. Consequently, in

985   addition to proliferation properties, mTOR is involved in metabolic reprogramming and

986   cytotoxic function of a recently activated CD8+ αβ T cells. Conversely, switching glycolysis

987   program to fatty acid oxidation operates the transition of effector T cells to memory cells

988   (*199*) and fatty acid oxidation was enhanced by rapamycin (*199*). As well, Araki et al. showed

989   that after acute LCMV infection, treatment with rapamycin increased the quantity and the

990   quality (CD127+KLRG1-CD62L+) of memory CD8+ αβ T cells. During the acute phase,

991   rapamycin increases the number of precursor memory cells, and during the contraction phase,

992   rapamycin induces better maintenance, enhancing the survival of antigen specific CD8+ αβ T

993   cells (*200*). Figure 16 summarizes the mTOR pathway and its upstream and downstream

994   signals in the naive and memory CD8+ αβ T cells. Finally, mTOR pathway has been poorly

995   studied in late effector T cells that are engaged during CMV infection, so its effects on their

996   proliferation, metabolism and cytotoxic functions are unknown.

997

998
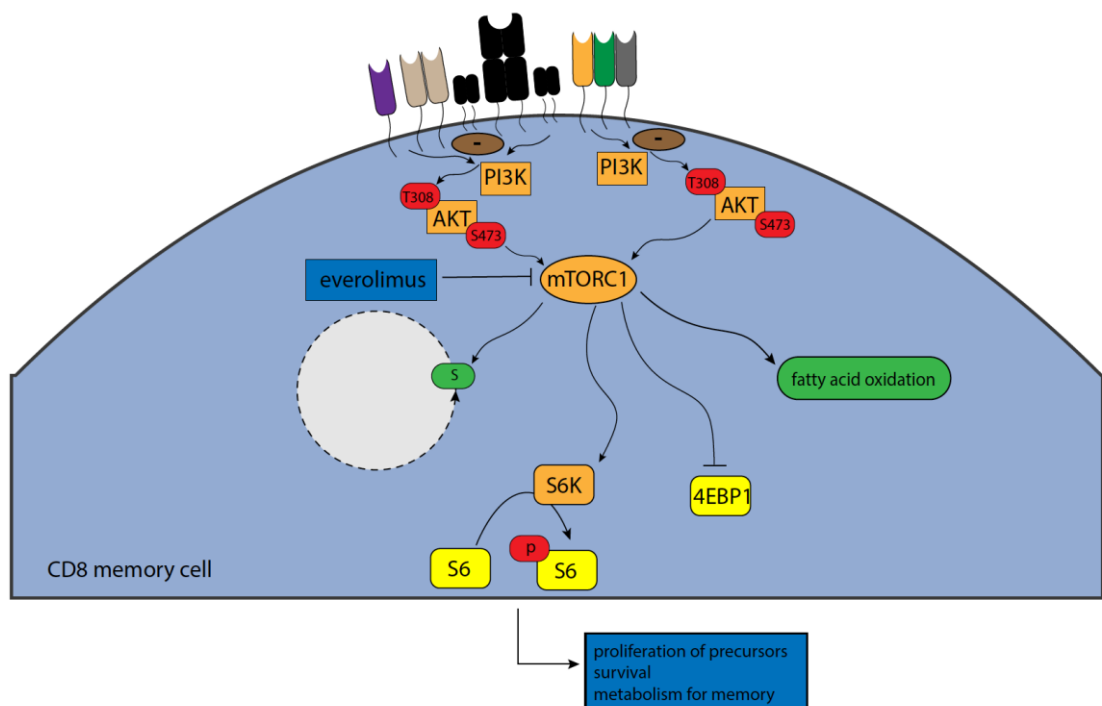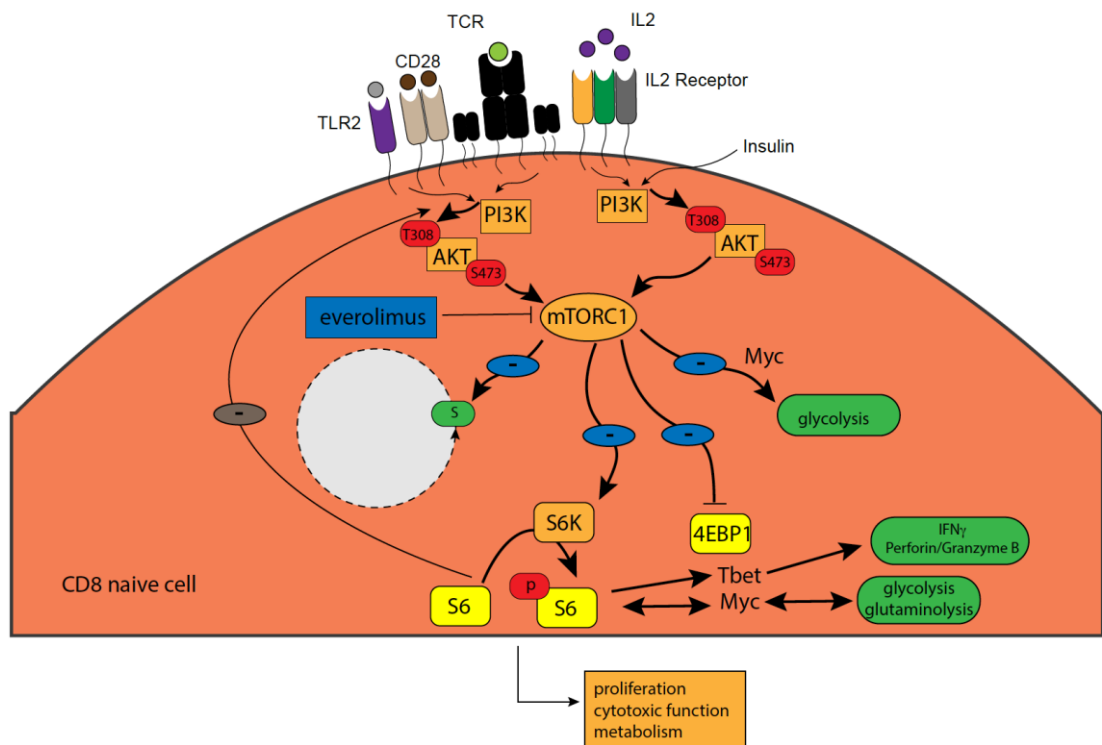
Figure 16 mTOR pathway in naive and memory CD8+ αβ T cells.

In recently activated naive cells, mTORC1 pathway activation drives positive signals for proliferation and cytotoxic functions. Blocking mTORC1 pathway with everolimus will decrease those effects on proliferation and functions. Conversely, memory cells need negative regulation of mTORC1 pathway for their better survival and proliferation of memory precursors. Everolimus in those memory cells will thus favor those functions.

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ii) Clinical evaluation of mTOR pathway inhibition regarding CMV incidence

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1005

1006 The role of mTORi in reducing the incidence of CMV infection has been documented since

1007 2011 (*201*). First, the positive effect of mTORi on CMV events was observed in *post-hoc*

1008 analyses of randomized trials including D+R- and R+ KTR, who received mTORi either in

1009 association with mycophenolate acid (MPA) (*202*), or with reduced dose of calcineurin

1010 inhibitors (*201, 203-206*). The primary endpoint of these studies was often to assess the non-

1011 inferiority of calcineurin reduced-dose immunosuppressive regimen. However, since the

1012 CMV event analysis was not initially anticipated as an outcome in these trials and therefore

1013 no systematic monitoring of CMV PCR was carried out, the impact on CMV could be

1014 underestimated. In addition, randomization in the different arms of these studies was not

1015 adjusted according to the use of prophylaxis *versus* preemptive therapy, which could have

1016 unbalanced the CMV occurrence. Later, three monocentric trials with CMV infection as a

1017 primary endpoint also showed a reduction of CMV infection or disease in patients receiving

1018 everolimus (Table 2) (*207-209*). R+ patients under preemptive strategy and with mTORi

1019 presented CMV infection between 10.4% and 13.6% and 7.9% of CMV disease whereas

1020 patients without mTORi presented CMV infection between 52.2% and 71.6% and 34.5% of

1021 CMV disease. Based on these studies, the last international guidelines for CMV management

1022 propose the use of mTORi as a potential approach to decrease CMV infection and disease in

1023 R+ KTR (*152*). These trials included heterogeneous patients for CMV serology, and for some

1024 of them, CMV infection was diagnosed with pp65 antigenemia, which is less sensitive for

1025 CMV detection than CMV PCR (*152*). Moreover, mTORi could be discontinued for adverse

1026 events (40% (*207*); 8.5% (*208*), 7% (*209*)) and no study has yet performed an ongoing

1027 treatment analysis to see the CMV incidence in patients still receiving mTORi. Finally, it

1028 remains unclear whether mTORi could be a third alternative as a preventive strategy in R+

KTR, thereby avoiding the drawbacks of preemptive strategy and universal prophylaxis. To better attest the validity of mTORi as an independent alternative of preventive strategy, the Bordeaux University Transplant Center conducted a multicenter French study under the supervision of L. Couzi to compare everolimus *versus* mycophenolic acid, in patients composed solely of R+ KTR, with DNAemia CMV and CMV disease as endpoints (EVERCMV study). Patients received either everolimus (EVR) or mycophenolic acid (MPA) in association with basiliximab, ciclosporin and prednisone, without universal prophylaxis but monitored with CMV PCR monitoring, as described above for preemptive strategy. The composite primary endpoint was CMV DNAemia, CMV treatment, graft loss, death, and discontinuation of the study 6 months after transplantation. 48.3% and 80.5% of patients in the everolimus and MPA groups reached the primary endpoint respectively (p<0.0001). Patients receiving everolimus showed a lower incidence of CMV DNAemia (39.2% vs. 77.8%, p<0.0001), received fewer antiviral drugs (21.8% vs. 49.4%, p<0.0002), and even if not significant exhibited less CMV disease (4.5 % *vs*. 11.3 %, p=0.2). The rate of adverse events was also similar in both groups, but everolimus was discontinued in 35.6% of patients for side effects. In a *post-hoc* analysis using a time-dependent Cox-proportional hazard regression model, exposition to everolimus was associated with a very low risk of CMV DNAemia (HR 0.14, CI95% 0.08-0.24) and of antiviral drug requirement (HR 0.08, CI95% 0.03-0.2). In a 12-month post-transplantation analysis, patients with ongoing everolimus treatment (n=56) experienced less CMV DNAemia (12/56, 21.4%), than the 31 patients with everolimus discontinuation (n=31) (19/31, 61.3%) and the MPA group (n=87) (61/87, 70.1%). Only 4 (7.14%) patients with ongoing EVR treatment experienced CMV DNAemia requiring treatment. On the opposite, patients with EVR discontinuation had a similar proportion of CMV DNAemia requiring treatment than the MPA group (36.4 and 46%, respectively). Compared to the proportions of CMV infection in R+ patients receiving other

preventive strategy in the literature, everolimus offers a lower incidence of CMV infection compared to preemptive strategy without everolimus (37% *vs* 78%), even more when everolimus is not stopped (21.4 vs 70.1%) and similar in comparison with a 3 month prophylaxis (11% *vs* 38%) (**DRAFT 3**).Those results showed that everolimus could replace either preemptive strategy or universal prophylaxis in R+ KTR in which as we said previously CMV disease occurs between 4% and 8% (*167, 210-212*) for 3 months-prophylaxis and between 7 and 13% (*167, 210-212*) for preemptive strategy as showed in Table 1.

iii) Mechanistic evaluation of mTOR pathway inhibition regarding CMV incidence

From a mechanistic point of view, we wondered how mTORi could reduce the incidence of CMV infection. As mentioned earlier, CMV uses the mTOR pathway to optimize its replication in the host cell and inhibiting mTOR inhibition could thus directly block CMV replication. However, mTOR inhibition was shown to result in viral cycle inhibition in macrophages but not in fibroblasts (*213*), suggesting that mTORi could not block viral replication in all cell subtypes. Moreover, other *in vitro* experiments have shown that depending on whether everolimus is cultured with fibroblasts before or after CMV infection, it may have a different impact on viral replication (*214*). When everolimus was incubated after CMV infection, it increased the number of infected-plaques, but reduced the size of infected-plaques without affecting virus yields and protein synthesis. When everolimus was used before CMV infection, the number of infected-plaques increased significantly and incrementally while the number of infected-plaques declined significantly with the duration of everolimus pre-treatment. However, under these conditions, everolimus was removed at the time of CMV infection. It is conceivable that the positive AKT feedback during everolimus treatment could lead to an even greater mTOR activity after the removal of everolimus, which

1079 would explain, the high infectivity associated with the duration of everolimus pre-treatment.

1080 Finally, when everolimus is cultured after CMV infection and maintained after infection, a

1081 decrease in DNA replication is observed after 4 days of culture, leading to a difference in the

1082 number of infected cells under everolimus condition from 5 days post-infection. However,

1083 although DNA replication and the number of infected-cell are reduced by everolimus, none is

1084 completely abolished (*214*). Moreover, the effect of drugs on *in vitro* cycle replication *(215)*

1085 does not prove its effect *in vivo*, as it had been claimed for the effect of hydroxychloroquine

1086 during COVID-19 as prophylaxis (*216*), early phase or active disease (*217, 218*). On the other

1087 hand, mTORi positive effect on CMV incidence in R+ and not in D+R- patients (*208, 209,*

1088 *219*) (as shown on Table 2) suggest that mTORi may act through the modulation of CMV-

1089 specific T cell response rather than through a direct antiviral inhibition. Indeed, previous

1090 studies showed that mTORi could also have implication on CMV-specific T cells. As

1091 previously said, mTOR pathway is induced in recently activated naive T cells, whereas it is

1092 downregulated in memory T cells, and its modulation is mostly unknown in effector CMV-

1093 specific T cells. In a very low number of patients, CMV-specific CD8+ αβ T cell counts was

1094 reportedly increased among everolimus treated patients between 6- and 12-months post-

1095 transplantation with no concomitant analysis of CMV events. Finally, everolimus in vitro

1096 showed a dose-dependent inhibition of allo-specific T cells as efficiently as ciclosporin or

1097 MPA but not on CMV-specific T cells which were strongly inhibited by MPA and ciclosporin

1098 (*220*). However, no mechanistic understanding was explored. Another study observed an

1099 improved functional effect of mTORi on CMV-specific T cells *in vitro* whereas alloreactive

1100 cells were inhibited but this study did not analyze the impact on CMV control (*221*).

1101 Nonetheless, CMV-specific population is supposed to be composed of highly functional

1102 effector memory T cells that remain at high level and induce good control of CMV infection.

1103 Consequently, it remains unclear why some R+ patients undergo severe CMV infection after

1104 transplantation and if their CMV-specific T cells are in a non-optimal state to control the

1105 virus. Thus, we supposed that the R+ patient who will undergo severe CMV replication

1106 present, at the time of transplantation, a CMV-specific T lymphocyte response which is not

1107 optimal. Secondly, we suppose that mTORi could positively impact this specific immune

1108 response. For this purpose, we took advantage of frozen PBMC resulting from the

1109 EVERCMV ancillary study. An extended phenotype was performed on γδ T cells and CMV-

1110 specific CD8+ αβ T cell on day 0 of transplantation. Next, we compared the profile of T cell

1111 populations in R+ KTR who will receive mycophenolic acid (MPA) and experience severe

1112 *versus* well-controlled infection or no CMV infection post-transplantation. Both αβ and γδ

1113 lymphocytes were shown to have a more dysfunctional phenotype (LAG3+, TIM3+, PD-1+,

1114 CD85j+) at day 0 in the 16 R+ KTR with severe CMV infection compared to the 17 KTR

1115 without or with spontaneously resolving CMV infection. In contrast, in patients treated with

1116 mTORi, we observed that the proportion of PD-1+ and CD85j+ αβ and γδ T-cells decreased

1117 when compared to MPA-treated patients, such as the frequency and severity of CMV

1118 infections. mTORi treatment also resulted in higher proportions of late-differentiated and

1119 cytotoxic γδ T-cells, IFNγ -producing and cytotoxic αβ T cells. *In vitro*, mTORi at low dose

1120 increased proliferation, viability and CMV-induced IFNγ production of γδ and αβ T-cells,

1121 decreased PD-1 and CD85j expression in the two subsets that shifted from

1122 EOMES^{high}Hobit^{low} to a more efficient EOMES^{low} Hobit^{high} transcription profile. In γδ T cells,

1123 this mTORi effect was related to increased TCR signaling (**ARTICLE 5**). Therefore, we

1124 observed that severe CMV replication is associated with a dysfunctional T-cell state and that

1125 mTORi improve T cell fitness in association with better control of CMV. As a mechanistic

1126 understanding, the effector T cells were found to have a high basal level of mTOR pathway

1127 activity. The reduction of this level with everolimus was associated with a better response to

1128 TCR, a more functional profile (PD-1^{low}CD85j^{low}EOMES^{low}Hobit^{high}), a better IFNγ

64

1129   production, proliferation and viability. Figure 17 summarizes our proposition on modulation

1130   of the mTOR pathway in effector memory T cells.

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1132
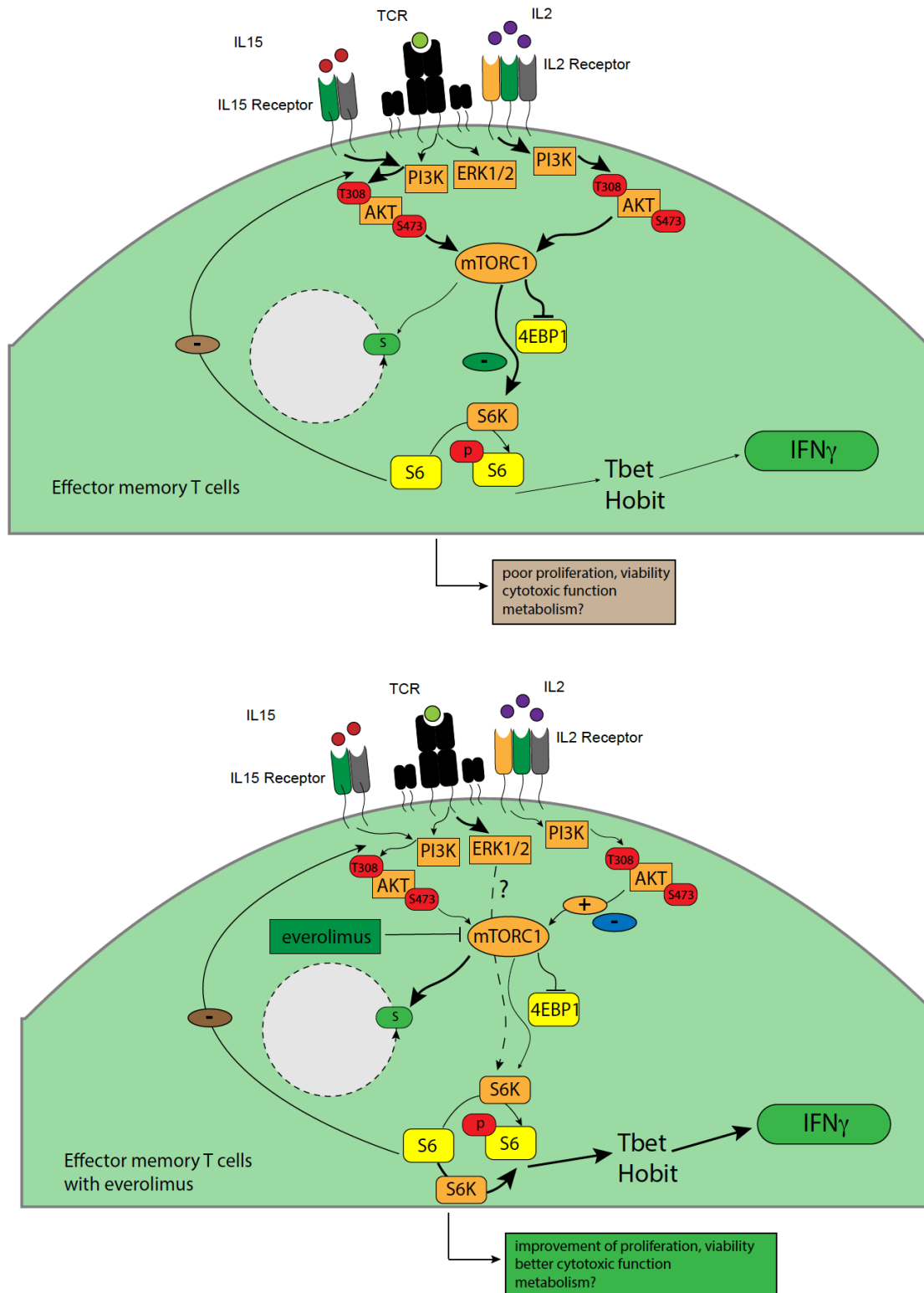
Figure 17 mTOR pathway in effector memory CD8+ αβ T cells

Effector memory T cells have a high level of mTORC1 activation (pS6 high), which is associated with low level of IFNγ, high level of Tbet and of Eomes and low level of Hobit. Decreasing mTORC1 pathway with everolimus (low pS6) leads to a better response to TCR signaling (pERK1/2), to a better induction of pS6 during TCR activation, to a decrease of Eomes and an increase of Hobit with a better IFNγ production. Moreover, everolimus lead to increase proliferation, viability.

V. Perspectives

A. Basic science perspectives

i) better understanding of γδ T cell role against CMV

Overall, we observed that CMV and its human host interact in complex relationships due to their long co-evolution during which CMV integrated host-homologous genes. Within the immune arsenal, γδ T cells harbor original features that may have led them to persist during this long-term coexistence with CMV. This particularity leads us to attempt to deepen the understanding of their physiology. Their recognition mode of CMV-induced stress molecules *via* their TCR can help them to get activated and overcome CMV evasion strategies and is one of their first originality. However, this is not the only way for them to recognize CMV-infected cells and the low number of identified TCR-antigenic ligands did not allow us to generalize their mode of recognition against CMV. Moreover, the observation of a third γδ T cell subset, $V\gamma9^{neg}V\delta2^{pos}$, persistent in adults, contributes to our awareness of the vast γδ T cell repertoire able to respond to CMV. However, except their difference from $V\delta2^{neg}$ γδ T cells regarding their expansion, we did not find other difference regarding their functions. We did not characterize much the type of Vγ and their involvement in the recognition of CMV-infected cells. Since Vγ9 has been involved in the innate binding of butyrophilin, so it will be interesting to do so and to see if the innate binding of Vγ chain plays a role in the activation and the localization of γδ T cells during CMV infection.

Finally, we will be interested in better characterizing the natural pool of γδ T cells present in naive individuals and able to recognize and get activated by CMV-infected cells (direction of the Master 2 project, F. Bos). We also have sorted CD69/CD71 double positive and double negative cells from culture with non-infected and CMV-infected fibroblasts to analyze the TCR repertoire of CMV-responding $V\delta2^{neg}$ γδ T cells and see the degree of clonality

compared to the non-responding cells. As a perspective, we will try to understand the TCR involvement in the CMV-recognition of those naive Vδ2^{neg} γδ T cells, in addition of the IL18 receptor and NKG2D. Altogether, we aim to better quantify and qualify this repertoire, to expand our knowledge about their different functions against CMV, and about how they recognize CMV-infected cells. We will see if this pool of natural γδ T cells with innate abilities to fight CMV may have original mode of recognition that place them at an important rank among CMV-immune response, if they display original counter-attacking CMV-escape. Moreover, we will try to understand their maturation trajectory to know if this natural repertoire of innate-like CMV-responding γδ T cells is able to switch to a memory phenotype and to confer long-term protection, which would also be an original fate.

ii) A better understanding of mTOR inhibition in CMV-specific T cells

We observed some effect of mTOR inhibition on CMV-specific γδ and αβ T cells on TCR signaling, pS6 induce during activation, inhibitory receptors, AKT feed-back, transcription factors regulating IFNγ production, IFNγ production during response to CMV and proliferation and viability. However, those observations could be completed in vitro by a better understanding of pathways involved in those improvements. For example, viability could be governed by the inhibition of pro-apoptotic regulators or by the activation of anti-apoptotic ones. In CD8+ αβ memory T cells, it has been shown that mTOR inhibition acts on Bcl2 (*200*). Secondly, it could be interesting to see that CMV-specific effector T cell fitness improvement induced by mTORi could be mediated by metabolic reprogramming. We tried to measure the basal glycolysis through lactate quantification in the supernatant of γδ T cells with or without everolimus and did not observed any difference. Other read-out could be used such as ATP production or oxygen consumption to see if equivalent glycolysis is operated with a higher level of ATP production or with a lower oxygen consumption.

1188   We observed also that characteristic modifications induced by mTORi on CMV-specific T

1189   cells were well corelated with better clinical CMV outcomes. However, to reinforce this link

1190   and to also discriminate the role of mTORi on immune response to its role on viral

1191   replication, it could be interesting to perform an animal model. To analyze the effect of

1192   mTORi on viral replication, we can infect CD3ε KO mice with CMV and treat those mice

1193   with or without everolimus and compare viral load and survival. If everolimus is efficient to

1194   inhibit CMV replication, we would see a difference with higher survival and lower viral load

1195   in the tissues of CMV-infected CD3ε KO mice treated with everolimus and conversely if no

1196   difference is observed, it would suggest that CMV infection is not better controlled through

1197   an antiviral effect of mTORi. If no difference is observed between CMV-infected CD3ε KO

1198   mice with or without everolimus, we also can analyze the effect of mTORi on γδ T cells and

1199   see if better outcomes on viral loads would be observed together with a higher functional

1200   profile of γδ T cells. We would also be able to analyze the dose-effect of everolimus to see if

1201   we observe a positive or negative effect on the control of CMV depending of the dose used

1202   and to confirm the effect of low-dose we observed in patients.

1203

1204   **iii) Evidence of dysfunctional CMV-specific T cells**

1205   We also observed that CMV-specific T cells that classically evolved in an immunocompetent

1206   host to an inflationary state could also evolved to a dysfunctional state that expose to clinical

1207   CMV reactivation during an immunosuppressed episode such as a kidney transplantation. The

1208   acquisition of this T cell dysfunctional profile is not clear. Hypothesis could be made by

1209   comparing with C-13 LCMV infection. The main difference between those two persistent

1210   viruses is that LCMV replicates chronically at a high level whereas CMV alternates latent

1211   phase and low level of replication. Those characteristics mainly explain why in the first case,

1212   specific immunity becomes exhausted and why in the second one, it becomes inflationary, as

it has been mentioned in the discontinuity theory of immunity (*222, 223*). Consequently, we can suppose that CMV could chronically and highly replicate in patients presenting immunosuppressive factors such as chronic kidney disease, previous exposure to immunosuppressive drugs (autoimmune disease, previous transplantation), diabetes and thus present "exhausted" CMV-specific T cells. However, we did not provide demonstrations to those potential explanations. For this purpose, a mice model with primary infection in which we can induce pharmacological immunosuppression and analyze the CMV viral load during reactivation and in parallel the functional kinetics of CMV-specific T cells would be of help to give answers. We can also test the association between CD85j and PD1 expression and the lower ability of CMV specific T cells to control the viral load and conversely the effect of blocking antibodies for CD85j and PD1 to see if a reinforcement of the response is observed.

B. Clinical perspectives

i) Stratifying CMV risk based on immunomonitoring to propose news therapeutic options

(1) In CMV negative patients :

Immunomonitoring showed that CMV-specific T cell tests (QuantiFERON, ELISPOT) help us mostly to avoid useless antiviral drug either for preventive or curative treatment when the test is positive. However, we still have no therapeutic option for patients for whom this test remains negative either after universal prophylaxis (*224*) with high risk of late-onset disease or after the course of a curative treatment of CMV disease (*225*) with high risk of recurrence and mutant-strain emergence.

In both of these scenarios, we tried to evaluate the potential benefit of mTOR inhibitors: (i) we observed no difference in term of CMV events when used as a preventive strategy in the literature (*219*), (ii) we did not obtain any reduction of the time to viral negativation, nor a

1239 decrease of viral/clinical recurrences when used during CMV disease treatment (**ARTICLE**

1240 **6**) (*226*).

1241 Thus, we can imagine that the negativity of CMV-specific T cell test would lead to either

1242 consider a decrease of immunosuppressive drugs to decrease the exposure to antiviral drug

1243 and thus decrease mutant strain emergence (**DRAFT 2**) or consider a new option: cellular

1244 therapy. The better knowledge about a large repertoire of CMV-responding $V\delta2^{neg}$ $\gamma\delta$ T cells

1245 in naive patients, could help us to rationalize protocols for expanding such $\gamma\delta$ T cells in these

1246 naive patients. Indeed, the expansion of these CMV-responding $\gamma\delta$ T cells with a natural

1247 repertoire could confer CMV protection after transplantation. This cellular therapy could

1248 become a new alternative in addition to antiviral drug to better prevent CMV disease, better

1249 manage CMV disease treatment by decreasing the duration of anti-viral drug, decrease viral

1250 and clinical recurrences and thus decrease the emergence of CMV mutant strain. Moreover,

1251 during the course of CMV disease, $V\gamma9^{neg}V\delta2^{pos}$ expansion as an immune-marker of severity

1252 could also be of help to decrease immunosuppressive treatment or propose cellular therapy in

1253 the future.

1254

1255 (2) In CMV positive patients

1256 Previous studies notably with IE1 ELISPOT showed that CMV preventive strategy could be

1257 avoided in R+ patients with a positive test at day 15 and interventional studies are undergoing

1258 to validate this strategy. In this work, we observed that a dysfunctional profile with high

1259 percentage of PD1+ and CD85j+ CMV-specific T cells measured at day 0 of transplantation

1260 was associated to a higher risk of CMV replication after transplantation and that mTORi

1261 could decrease the percentage of those cells with clinical outcome improvements. However,

1262 those results have to be moderated. First, the percentage of cell expressing inhibitory

1263 receptors was significantly different between patients with or without severe CMV replication

1264 after transplantation but it was done on a low number of patients and has to be reproduced on

1265 an external cohort. Moreover, if results are confirmed, a threshold of CD85j+ and PD1+ cells

1266 will have to be established in order to consider it as a biomarker. Then, this dysfunctional

1267 profile of PD1+ CD85j+ could represent a new biomarker to predict post-transplantation

1268 infection risk in R+ KTR, combined for example with IE1-ELISPOT and will have to be

1269 tested in an interventional study. If the patient presents an efficient profile of T cell response

1270 with a positive IE1 ELISPOT and a low percentage of CD85j+ and PD1+ CMV-specific T

1271 cells, neither preemptive strategy nor prophylaxis should be required and we thus will avoid

1272 useless PCR surveillance or toxic antiviral drug for those patients (proposition in Figure 18).

1273 On the other side, if we observed a dysfunctional CMV-specific T cell profile, we can stratify

1274 patients who should benefit from mTORi treatment or preventive strategy (proposition in

1275 Figure 19).

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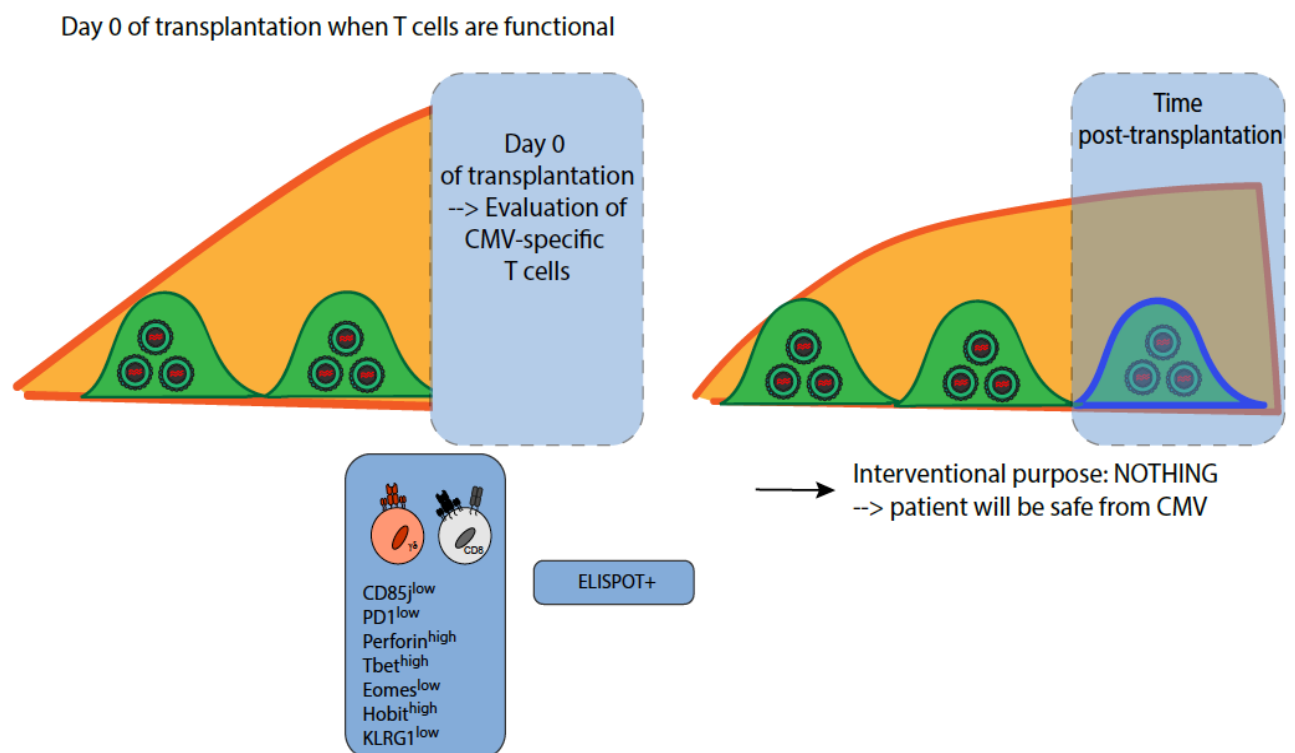
1277 *Figure 18 Proposition stratifying R+ patients based on CMV-immune monitoring when T cells are functional.*

1278 *If CMV-specific T cells present a functional profile at day 0 of transplantation, even if their function could*
1279 *decrease with immunosuppressive drug, they should maintain enough to control CMV replication and CMV*
1280 *preventive strategy could be avoided.*

Figure 19 Proposition stratifying R+ patients based on CMV-immune monitoring when T cells are dysfunctional

If CMV-specific T cells present a dysfunctional profile at day 0 of transplantation, mTORi could be provided as preventive strategy, the functions of T cells would be improved enough to control CMV replication and CMV preventive strategy could be avoided. Alternatively, the control of CMV could be obtained by antiviral drug either with the preemptive or prophylactic approach

C. Conceptual perspectives

i) Operationalizing exhaustion

We have observed that classically CMV-specific T cells harbor an inflationary state as opposed to LCMV specific cells which have been described as harboring an exhausted state. However, in some patients, a "dysfunctional" profile has been observed which correlate with higher risk of high CMV replication. As we mentioned previously, specific T cells in the long term of viral infection could become inflationary or exhausted because of the intensity of viral replication or in any case, it was suspected when we read the literature about exhaustion during LCMV infection. However, we observed that three distinct approaches to define immunological exhaustion co-exist and that they only partially overlap, generating potential misunderstandings. Indeed, the founding period for modern uses of the notion of exhaustion was the 1990s, during which exhaustion was defined both mechanistically and functionally. For Zinkernagel and his group (*227*) as well as Ahmed and his group (*228*), "exhausted" T cells are cells that, exposed chronically to high quantities of antigen, are activated and proliferate, before becoming dysfunctional. In other words, exhaustion was defined both by dysfunction (these T cells fail to do what effector T cells are expected to do, namely eliminate the virus) and by a double mechanism (high viral load and chronicity). In subsequent research until present-day, three approaches have co-existed, with often unclear connections. The first approach defines as exhausted the cells that are produced by a given *mechanism* (typically, but not necessarily, chronic exposure to a high load of antigen). The second approach defines as exhausted the cells that present the same cellular *dysfunction* (typically, the absence of an expected effector response). Finally, the third approach defines as exhausted the cells that present the same *molecular markers* (typically, PD-1). One difficulty is that authors do not always say which approach they have in mind when they qualify cells as "exhausted". A second, even more, serious difficulty is that authors often do as if these three approaches

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necessarily aligned (i.e., as if the three properties always occurred together), when in fact they

don't. In our work, we give several examples of non-overlap, and we insist on the importance

of both distinguishing and combining these three approaches to build a precise and

operational account of exhaustion. In our view, the lack of articulation between the three

approaches distinguished above is the prime explanation for the current ambiguities and

disagreements around the notion of exhaustion, as exemplified recently in the various and

often competing positions expressed by 19 experts (*229*). The three approaches to

immunological exhaustion often do not converge. Some situations can be described as

exemplifying "exhaustion" if seen from the functional viewpoint, but are not associated with

the classic molecular markers of exhaustion and/or the classic mechanisms of exhaustion.

From the 1990s to the present day, many immunologists working on exhaustion have

suggested that exhaustion might in fact reflect an evolutionary conserved mechanism of

immunoregulation (*230, 231*). What we, as observers, have dubbed "exhaustion" to initially

describe cells that seem to fail to realize their expected effector functions may well actually

be one instance of a beneficial physiological process, preventing excessive immune responses

and excessive damages (**DRAFT 4**).
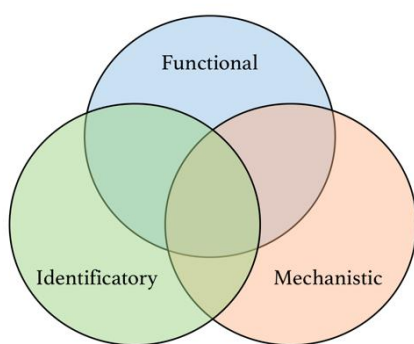
Figure 20 Partial overlapping of the three criteria used to define exhaustion.

Identificatory, functional and mechanistic characteristics are used to define exhaustion but only partially overlap.

This connection between exhaustion and immunoregulation is interesting and fruitful, because the truth is that, in most experimental and clinical situations, what we are interested in and are trying to achieve is immunoregulation: Novel major experimental and therapeutic challenges could be raise by this connection :

- Why not examine exhaustion in the context of tolerance to the "self"? Is it, in that case, a form of self-regulation?

- Why not examine exhaustion in the context of tolerance to microbiota?

- Negative consequences of excessive limitation of exhaustion in contexts of infection: although limiting exhaustion in some contexts of viral infection is a legitimate objective, we expect too strong an inhibition of exhaustion to lead to pathological consequences, from immunopathology to the development of autoimmune disorders. In the view of effector function to obtain CMV viral control, inflationary state seems the optimal way and conversely, exhaustion would lead to a weaker control of the virus. However, the previous reflection in which exhaustion could be beneficial at the level of the organism, preventing immunopathology could highlight a different way to interpret the long-term equilibrium between CMV and its host. Indeed, as described also by Medhzitov, this dysfunctional state observed in some patients could constitute the ability of the host to tolerate a pathogen's presence and thus constitute a distinct host defense strategy. This host strategy has been called "disease tolerance" as opposed to "resistance" in which the host increase his host defense to try to eliminate the pathogen. The choice between the "disease tolerance" and the resistance could rely on the host-fitness, which mean what would cost the most to the host (*232*). So in a way, this dysfunctional state observed in some patients could be the one what costs the least to the organism and prevent immunopathology generated by the chronic activation of immune

1361   response to CMV. The high fitness of the host could be measured by its ability to choose

1362   between both strategies. However, it could be even more complex when the host become

1363   immunocompromised and has to revert to resistance strategy to better fight the virus which

1364   become in this situation more pathogen than the pathogenicity of host-defense.

1365   Immunosuppression could thus be defined as a decrease of host-fitness during which the host

1366   could   not   choose   to   revert   from   disease   tolerance   to   resistance.

VI.   References

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VII. Annexes

A. Articles

i) **Article 1 Understanding human γδ T cell biology toward a better management of cytomegalovirus infection (accepted)**

ii) **Article 2 Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2 (accepted)**

iii) **Article 3 Characterization of a unique γδ T-cell Subset as a specific marker of cytomegalovirus infection severity (accepted)**

iv) **Article 4 Single-cell RNA sequencing unveils the shared and the distinct cytotoxic hallmarks of human TCRVδ1 and TCRVδ2 γδ T lymphocytes (accepted)**

v) **Article 5 mTOR inhibitors prevent CMV infection through restoration of functional αβ and γδ T cells in kidney transplant recipients (submitted)**

vi) **Article 6 Effect of mTOR inhibitors during CMV disease in kidney transplant recipients: Results of a pilot retrospective study (accepted)**

B. Drafts of article

i) **Draft 1 Identification of a natural repertoire of innate-like human γδ T cells reactive to CMV**

ii) **Draft 2 Characteristics and time course of CMV infections leading to antiviral drug resistance**

iii) **Draft 3 Evaluation of everolimus as a third preventive strategy of CMV disease in seropositive transplant recipients**

iv) **Draft 4 Immunological exhaustion: How to make a disparate concept operational? (submitted to the Journal of experimental medicine)**

INVITED REVIEW

Understanding human γδ T cell biology toward a better management of cytomegalovirus infection

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Abstract

Cytomegalovirus (CMV) infection is responsible for significant morbidity and mortality in immunocompromised patients, namely solid organ and hematopoietic cell transplant recipients, and can induce congenital infection in neonates. There is currently an unmet need for new management and treatment strategies. Establishment of an anti-CMV immune response is critical in order to control CMV infection. The two main human T cells involved in HCMV-specific response are αβ and non-Vγ9Vδ2 T cells that belong to γδ T cell compartment. CMV-induced non-Vγ9Vδ2 T cells harbor a specific clonal expansion and a phenotypic signature, and display effector functions against CMV. So far, only two main molecular mechanisms underlying CMV sensing have been identified. Non-Vγ9Vδ2 T cells can be activated either by stress-induced surface expression of the γδT cell receptor (TCR) ligand annexin A2, or by a multimolecular stress signature composed of the γδTCR ligand endothelial protein C receptor and co-stimulatory signals such as the ICAM-1-LFA-1 axis. All this basic knowledge can be harnessed to improve the clinical management of CMV infection in at-risk patients. In particular, non-Vγ9Vδ2 T cell monitoring could help better stratify the risk of infection and move forward a personalized medicine. Moreover, recent advances in cell therapy protocols open the way for a non-Vγ9Vδ2 T cell therapy in immunocompromised patients.

KEYWORDS
CMV, immunomonitoring, immunotherapy, transplantation, γδ T cells

1 | CMV: CURRENT ISSUES AND POPULATIONS AT RISK

Cytomegalovirus (CMV) is a 200- to 300-nm virus, which belongs to the beta herpes virus family. CMV seroprevalence ranges from 40% in Canada to 90% in Brazil and China.[1] Whereas it is almost asymptomatic in immunocompetent individuals, it is responsible for significant morbidity and mortality in immunocompromised patients and pregnant women. In solid organ transplant recipients (SOTR), hematopoietic cell transplant recipients (HCTR), or human immunodeficiency virus (HIV) patients, CMV causes a multiorgan disease with a large clinical spectrum, which has been well defined recently in the CMV Drug Development Forum.[2] During pregnancy, CMV

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primary infection of naive pregnant women can induce congenital infection, often leading to sensorineural hearing loss and neurological and cognitive impairments in newborns.

In 2018, 150 000 solid organ transplantations were registered by transplant programs worldwide including kidney, liver, heart, lung, pancreas, and small bowel transplantations. Despite prevention strategies based on antiviral treatment (valganciclovir), CMV-seronegative recipients receiving an organ from a seropositive donor (D⁺R⁻) have the highest risk of developing CMV disease (20%), followed by CMV-seropositive recipients (R⁺, 10%).[3,4] Indirect effects of CMV infection in these patients are also observed including rejection, cardiovascular events, and allograft loss.[5]

In 2016, 90 000 hematopoietic cell transplantations were reported.[6] Among them, up to 80% of R⁺ and 30% of R⁻ recipients can be affected by CMV infection.[7] With the current prevention strategies, the incidence of cytomegalovirus disease is between 5% and 10% in real-world practice.[8] With the available prevention strategies, CMV infection is still associated with an increased mortality in allogeneic hematopoietic cell transplantation.[9]

In 2016, 19.5 million of the 36.7 million patients living with HIV in the world had access to treatment (unaids.org; global report 2019). CMV retinitis was the most common opportunistic ocular infection in patients with acquired immunodeficiency syndrome (AIDS) and a CD4⁺ T cell count below 50 cells/ml. Since its prevention relies on the control of HIV infection, bilateral blindness from CMV retinitis is continuously decreasing from 14.8/100 person-years in the pre-antiretroviral therapy era to 0.4/100 person-years in the era of the antiretroviral therapy.[10]

Cytomegalovirus is the most common cause of congenital infection and non-genetic sensorineural hearing loss. Among the 150 million pregnancies each year, prevalence of congenital CMV infection is comprised between 0.2% and 2%. It occurs in 30% of seronegative women developing CMV primary infection during pregnancy. Non-primary infection can also occur in young women. It accounts for up to 25% of congenital sensorineural hearing loss, and can cause developmental delay, cognitive impairment, cerebral palsy, epilepsy, impaired vision function, and autism spectrum disorder.[11-13]

For all these vulnerable people at risk of CMV disease and CMV-associated morbidity or mortality, there is an unmet need for safe and effective CMV treatment. We have learned from basic and clinical studies that fighting CMV required first an antiviral therapy to inhibit or decrease CMV replication, and then the establishment of a persistent CMV-specific immune response, able to control the virus lifelong to avoid recurrence. Today, compelling evidence shows that the monitoring of CMV-specific immune response could predict either the absence or the resolution of CMV infection in SOTR and HCTR. Since it is now feasible to expand ex vivo CMV-specific lymphocytes, CMV cell therapies could also take a central place in the therapeutic arsenal for fighting against CMV in the future.

2 | IMMUNE RESPONSE TO CYTOMEGALOVIRUS INFECTION

2.1 | Viral entry, dissemination, and reactivation

Natural entry route has been well studied using the murine CMV. In this model, CMV enters the host via the respiratory tract, especially the nose. CMV host entry requires non-specific binding to heparan-positive epithelial surfaces.[14] Then, dendritic cells and monocytes become infected and migrate to new tissues. Among them, the main site of long-term murine CMV production could be salivary gland acinar cells.[15,16] Human CMV has a large tropism and can infest different types of cells such as fibroblasts, endothelial and epithelial cells, monocytes, CD34⁺ cells, granulocytes, and dendritic cells (DCs).[17] CMV entry into epithelial and endothelial cells requires a multiproteic molecular complex involving CMV gene products UL131A-UL128 associated with the gH and gL glycoproteins. The entry into fibroblast requires only the gH/gL/gO (or gH/gL) complex.[18]

After primary infection, the viral genome persists for the host lifetime in a latency state in monocytes,[19] dendritic cells,[20] endothelial cells, and CD34⁺ cells.[21,22] A key hallmark of latency is the suppression of CMV immediate early (IE) gene expression.[23]

In SOTR, a "two-hit" process involving the reactivation of CMV (first hit) and then the dissemination of the virus (second hit) is necessary for inducing CMV disease.[24] CMV reactivation can occur during inflammatory or infectious events. In the context of transplantation, transcriptional reactivation of CMV can be initiated after ischemia/reperfusion lesions[24] or allogenic stimulation.[25] Key mediators of CMV reactivation are tumor necrosis factor-α (TNF-α), catecholamine, and prostaglandin, which activate IE gene expression.[17] The second hit is given by immunosuppression. Importantly, immunosuppression alone does not induce viral reactivation, but facilitates CMV dissemination to other organs by inhibiting the immune response against the virus.[24]

2.2 | Main immune effectors against CMV

The control of CMV infection requires a close cooperation between innate and adaptive immune effectors. However, the coevolution of CMV within its host resulted in the emergence of immunomodulatory molecules encoded by the virus, also called immunoevasins. These molecules are able to escape at least four categories of immune activating pathways implemented for controlling viral infection. They can modulate and inhibit antigen presentation, promote escape from missing-self recognition, downregulate the ligands of activating natural killer (NK) cell receptors, and target Fc receptors for inhibiting Fcγ receptors activation and antibody-dependent cell cytotoxicity (see ref. 14 for a review). To bypass all these strategies used by CMV to unsettle immunity, the host recruits no less than five effectors: NK cells, γδ T cells, immunoglobulin G (IgG), CD8⁺ T cells, and CD4⁺ T cells.

Neutralizing CMV IgG targets gB and gH surface glycoproteins,[26-28] and can limit viral dissemination.[29] However, they seem to

play a minor function in controlling infection since B cell–deficient mice control rapidly CMV infection without subsequent reactivation,[30] and treatment with CMV hyperimmune IgG alone is not able to prevent efficiently CMV disease in SOTR.[31]

In mouse models using lymphocyte depletion, it has been elegantly demonstrated that no cell subset was essential to control CMV infection or reactivation, highlighting a robust redundancy of CMV cell responses[30,32] However, human studies in SOTR and HCTR have demonstrated the central role of cell-mediated immunity, especially CMV-specific CD8^{+} T cells and γδ T cells for avoiding CMV recurrence.[33,34] Four cell types are mobilized against CMV, two belonging to innate immunity (γδ and NK cells) and two belonging to adaptive immunity (CD8^{+} and CD4^{+} T cells). Recent studies have highlighted that both CMV-induced γδ T and NK cells shared properties belonging to adaptive immunity, such as clonal expansion and memory.[35,36] In the following paragraph, we will briefly describe the main characteristics of the response to CMV by NK cells, and CD8^{+} and CD4^{+} T cells, while γδ T cells will be extensively developed.

2.3 | NK cells

NK cells are considered as one of the most prevalent actors of anti-CMV innate immunity.[30,37] In humans, NK cell deficiency disorders are associated with severe, including fatal, viral infections, with particular susceptibility to herpesviral infections, such as cytomegalovirus.[38] After kidney transplantation, NK cell cytotoxicity potential increases during CMV infection and is correlated with circulating NK cell numbers.[39] NK cell reactivity against CMV-infected cells is the result of a balance governed by the activation of various receptors that sense alterations in the expression of ligands on the surface of CMV-infected cells.[40] An increase in NK-activating receptors could confer to the host a better protection against CMV infection.[41] NK cell clonal expansion is observed after CMV infection and leads to the development of an immunological memory,[42] mimicking the antigen-driven expansion of CD8^{+} T cells.[35] CMV-seropositive healthy human individuals exhibit an expanded population of NK cells expressing the activating CD94/NKG2C receptor, compared with seronegative individuals.[43,44] A recent study found a viral ligand for NKG2C, namely CMV-encoded UL40 peptides loaded onto human leukocyte antigen E (HLA-E), and proposed that these peptides could induce the proliferation and differentiation of a subset of adaptive NKG2C^{+} NK cells,[35,45] suggesting that this response was clonal. Finally, NK cells could exert an antibody-dependent cell cytotoxicity against CMV-infected cells, through CD16 (FcγRIIIA) activation by CMV IgG.[46,47]

2.4 | CMV-specific CD8^{+} T cells

In CMV-seropositive individuals, CMV-specific CD8^{+} T cells represent 4.6% and 10.2% of total and memory CD8^{+} lymphocyte pools, respectively.[48] After primary infection, CMV-specific CD8^{+} T cells can be detected from 20 days after the occurrence of viremia in SOTR,[49] and their number can even increase after reactivation in R^{+} SOTR.[50]

During primary infection, they exhibit an antigen-driven early-differentiating phenotype: CD27^{+}, CD28^{+}, CD45RO^{+}, CD45RA^{−}, CCR7^{+}, perforin^{+}, granzyme B^{+}, Ki67^{++}.[49,51] At the chronic phase of infection, after viral clearance, there is a substantial expansion of CMV-specific CD8^{+} T cell memory pools over time—a process described as memory inflation,[52] that is to say that, conversely to a conventional phase of contraction after viral clearance, a high number of CMV-specific T cells with an effector and functional memory phenotype persist in the long term. At this time, two subsets of CMV-specific CD8^{+} T cells are found in the peripheral blood of patients: a classical central memory population CD27^{+}, CD28^{−}, CD45RO^{+}, perforin^{+/−}, granzyme A-B^{+/−}, CCR7^{−}, CD127^{+} (IL7Ralpha), also observed in other chronic infections (hepatitis C virus HCV and HIV), and those able to proliferate upon T cell receptor (TCR) stimulation, which has a low cytotoxic potential. The most prominent CMV-specific CD8^{+} T cells are the terminally differentiated effector memory cells (or TEMRA cells), which represent up to 75% and 50% of CMV-specific CD8^{+} T cells in R^{+} SOTR and healthy individuals, respectively.[53] They are CD27^{−}, CD28^{−}, CD127^{−}, CD45RA^{+}, perforin^{++}, granzyme A-B^{++}, CCR7^{−}, CD62L^{−}, CX3CR1^{+}, and CD57^{+}. This TEMRA subset is CMV-specific,[51,54] and has low proliferation ability upon TCR stimulation but a huge cytotoxic potential.[55,56] These cells also harbor a large panel of NK receptors.[57] The sustained expansion of CD8^{+} T cells with a TEMRA phenotype is a consequence of low-level antigen persistence through sporadic viral reactivation events.[52] In mouse, adoptive transfer of central memory MCMV-specific CD8^{+} T cells into latently infected hosts resulted in T cell proliferation and differentiation into late effector cells.[58] In this mouse model, the large pool of these cells found in blood and tissues was rapidly resupplied from central memory cells and naive precursors.[52,58]

Antigen presentation by infected cells requires additional signals from co-stimulatory molecules such as 4-1BB, OX40, and CD70, and cytokines such as IL-2 or IL-15, in order to induce T cell proliferation and differentiation into TEMRA cells.[52] During primary infection, the overall CD8^{+} T cell population is highly polyclonal,[59] whereas at the chronic phase, it is often dominated by only a few epitope-specific clones.[60] Furthermore, public TCRs can be detected within CMV-specific CD8^{+} T cell populations from different individuals.[61] Interestingly, maintenance of a highly diverse αβTCR repertoire is correlated with improved control of CMV.[61] CMV peptides from the three following open reading frames (UL48, UL83, and UL123) are recognized by more than half of individuals.[48] Interestingly, IE-1 (UL123)-specific CD8^{+} T cells are associated with less CMV reactivation in SOTR,[62,63] probably because UL123 is the first CMV protein to be expressed in infected cells. In vitro, CMV-specific CD8^{+} T cells are able to exert antiviral effector functions trough interferon-γ (IFN-γ) production. They can also kill autologous cells loaded with CMV peptides or CMV-infected cells.[49,64]

In mouse models, late effector CD8^{+} T cells maintain long-term control of viral replication.[52] Nevertheless, the depletion of CD8^{+} T cells alone is not sufficient to induce CMV reactivation. However,

the depletion of both CD8$^+$ T and NK cells is associated with more CMV reactivation than the depletion of CD4$^+$ and NK cells.[30] Taken together, these data support the idea that CD8$^+$ T cells play a central role in the hierarchy of cells involved in the control of CMV.

2.5 | CMV-specific CD4$^+$ T cells

In humans, CMV-specific CD4$^+$ T cells represent 4% and 9.1% of total and memory T lymphocyte pools, respectively.[65] After a primary infection, CMV-specific CD4$^+$ T cells can be detected from 7 to 10 days after the occurrence of viremia in SOTR,[66] and a delay in CD4$^+$ T cell expansion is correlated with the severity of the disease.

Cytomegalovirus primary infection leads to the rapid emergence of a specific population of CD4$^+$ CD28$^-$ granzyme B$^+$ cells, easily identifiable in the peripheral blood.[67] At the chronic phase of infection after viral clearance, they acquire an effector memory phenotype close to the one observed in CD8$^+$ T cells: CD27$^-$, CD28$^-$, CD45RA$^-$, CD45RO$^+$, CD62L$^-$, CCR7$^-$, CD11a$^+$, CD11b$^+$, intercellular adhesion molecule 1 (ICAM-1)$^+$, granzyme A-B$^+$, perforin$^+$, Ki67$^-$, and CD57$^+$.[66-68]

Cytomegalovirus peptides from the five following open reading frames (UL55, UL83, UL86, UL99, and UL122) are recognized by more than half of individuals.[48]

CD4$^+$ T cells play a central role in anti-CMV immunity by helping B cells to mount a specific humoral response against CMV antigens [69] and CD8$^+$ T cells to perform their effector functions. They also produce IFN-γ, TNF-α,[67] and MIP-1β, express CD107a after activation by CMV peptides, and induce the lysis of cells loaded with CMV peptides.[67-69]

3 | γδ T CELL INVOLVEMENT IN THE ANTI-CMV IMMUNE RESPONSE

The T cell compartment is composed of conventional αβ T cells, as well as γδT cells. In humans, γδT cells are classically divided into two main subsets, based on their γ and δTCR chain expression. Vδ2-positive γδ T cells, expressing a δ2 chain, are opposed to Vδ2-negative γδT cells. More recently, a subset of Vγ9negVδ2pos γδ T cells was identified in fetuses [70] and subsequently in adults [71,72] and appears to be closer to the Vδ2neg subset regarding TCR diversity and phenotype. These observations led to a new γδ T cell classification, in which Vγ9posVδ2pos γδ T cells or Vγ9Vδ2 γδ T cells are currently opposed to non-Vγ9Vδ2 γδ T cells, which gather both Vδ2neg γδ T cells and Vγ9negVδ2pos γδ T cells.[72] This classification also recapitulates exclusive biology, antigen recognition, and pathologies in which they are involved. On the one hand, Vγ9Vδ2 T cells sense variations in cellular production of phosphorylated metabolites of the isoprenoid pathway (called phosphoantigens). The most active phosphoantigens are produced by microorganisms such as *Mycobacterium tuberculosis*, *Plasmodium falciparum*,[73] or *Toxoplasma gondii*.[74] Less active endogenous phosphoantigens can also accumulate into host cells upon

activation or transformation.[75,76] On the other hand, non-Vγ9Vδ2 T cells harbor a dual reactivity against cancer and CMV-infected cells. In those contexts of infected and transformed cells, non-Vγ9Vδ2 T cells recognize stress-induced antigens.

Initially, the involvement of Vδ2neg T cells in the anti-CMV response was highlighted in immunocompromised patients.[77,78] Indeed, CMV infection in an immunocompetent host is usually asymptomatic due to the establishment of a robust and specific immune response. Moreover, after primary infection, all immune effectors contribute to inhibit virus reactivation,[17] thus preventing studies on acute primary infections in healthy individuals. Nevertheless, Vδ2neg T cell response to CMV in immunocompetent individuals has been evidenced through their increased percentages and late-differentiated phenotype in CMV pre-exposed individuals, that is, CMV-seropositive donors.[36,79,80] However, CMV becomes invasive and leads to disease in peculiar situations of αβ T cell deficiencies either because of immaturity,[70] congenital immunodeficiency,[81,82] or induced immunosuppression by immunosuppressive drugs [77,78,83-85] either in solid organ or in bone marrow transplantation. In these clinical settings, important needs remain in order to better control this life-threatening disease. In this review, we describe how a better understanding of non-Vγ9Vδ2 T cell biology can help improve both CMV prevention and treatment and how these cells can be harnessed for both immunomonitoring and cell therapy.

In order to harness non-Vγ9Vδ2 T cell properties for a clinical use in the CMV setting, it seems essential to provide strong arguments for: (a) their efficient implication in the response against CMV and (b) their specificity for this virus. Indeed, while CMV-specific αβ T cells [48] directly recognize CMV-derived peptides, the direct recognition of viral components by non-Vγ9Vδ2 T cells has not been observed so far, since they recognize self-encoded, stress-induced antigens (see below). Nevertheless, five major observations suggest that non-Vγ9Vδ2 T cells respond specifically to CMV (Figure 1).

3.1 | Specific expansion of non-Vγ9Vδ2 T cells in CMV-infected individuals

Initially, a longitudinal expansion of non-Vγ9Vδ2 T cells was observed in the peripheral blood of kidney transplant recipients (KTR) undergoing CMV infection.[77,78] This observation was later extended to other immunocompromised settings such as newborns,[70,86-88] HCTR,[83,85,89-91] and liver-,[92-95] lung-,[80,95] heart-, and intestine-transplanted patients.[95] Besides, increased numbers of non-Vγ9Vδ2 T cells have also been described in the peripheral blood of CMV-seropositive healthy donors.[36,80] Interestingly, Vδ2neg γδ T cells and Vγ9negVδ2pos γδ T cells behave differently in KTR during the course of CMV infection since only Vγ9negVδ2pos T cell expansion was correlated with the severity of CMV, regarding symptoms and CMV DNAemia duration.[71]

Moreover, in CMV-seropositive patients with end-stage liver diseases (primary sclerosing cholangitis, primary biliary cholangitis, alcoholic liver disease, non-alcoholic steatohepatitis, hepatitis
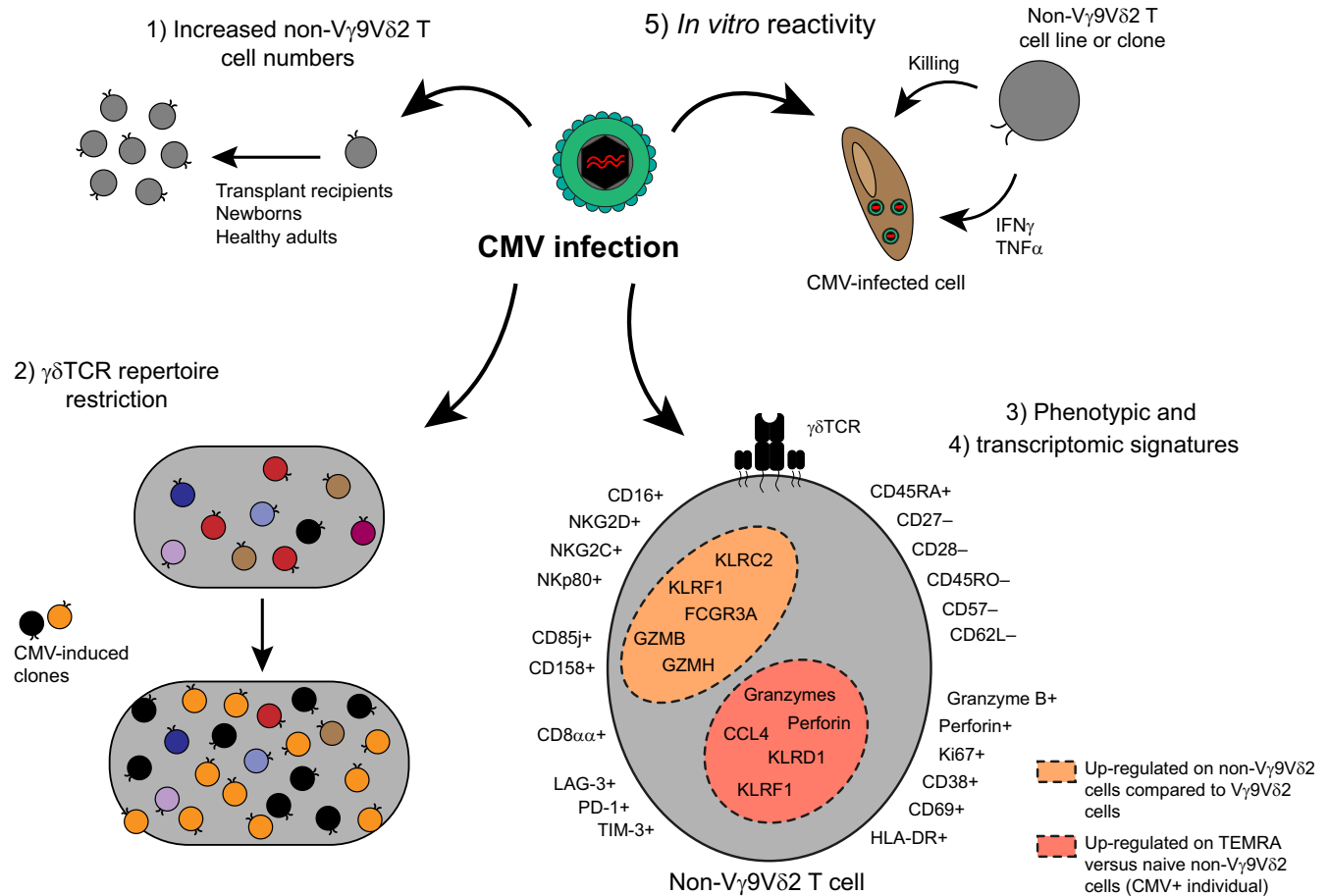
FIGURE 1 Non-Vγ9Vδ2 T cell involvement in the anti-cytomegalovirus immune response. Several clinical observations suggest that non-Vγ9Vδ2 T cells respond to CMV infection in vivo. They expand following infection in the peripheral blood of solid organ transplant recipients, hematopoietic cell transplant recipients, newborns, and healthy donors. This expansion is clonal and associated with a restriction of the γδTCR repertoire. CMV-induced non-Vγ9Vδ2 T cells harbor a specific transcriptomic and phenotypic signature composed notably of TEMRA markers, activation and cytotoxicity markers, checkpoint inhibitors, and NK receptors. In vitro, non-Vγ9Vδ2 T cells produce pro-inflammatory cytokines and kill CMV-infected target cells

C virus, and hepatitis B virus), increased numbers of peripheral blood and liver-infiltrating Vδ1+ γδ T cells were also described.[96] This observation that Vδ1+ γδ T cells are enriched in CMV-positive versus CMV-negative liver explants indicates that this subset may contribute to unconventional T cell immune surveillance of CMV-infected tissues, similar to what was previously described in mice.[97]

Importantly, no any other viral infection, including viruses belonging to the herpesviridae family, such as herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein-Barr virus (EBV), but also influenza,[77,98] could be associated with non-Vγ9Vδ2 T cell expansion. EBV-induced reactivity must be interpreted with caution. Indeed, Vδ1 T cells proliferate when co-cultured with B-EBV-transformed B cell lines in vitro [99] but it is not EBV-specific since this proliferation has also been observed with EBV-uninfected transformed B cell lines.[100,101] Also, γδ T cell expansion has been observed in the peripheral blood of patients during the acute phase of EBV infection but this expansion only concerns the Vγ9Vδ2 compartment.[102,103]

3.2 | TCR repertoire analysis of CMV-induced non-Vγ9Vδ2 T cells suggests an adaptive response

Focusing on the TCR repertoire of non-Vγ9Vδ2 T cells provides a second line of evidence for a CMV-specific amplification. The γδTCR repertoire is the result of the rearranged TRG and TRD loci, which code for the TCRγ and TCRδ chains, respectively. A first level of diversity is provided by all the different Vδ and Vγ segments that can associate with Cδ and Cγ segments, respectively, and called combinatorial diversity. In non-Vγ9Vδ2 T cells, eight Vδ regions (Vδ1 to Vδ8) and six Vγ regions (Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, and Vγ9) can be expressed. Intriguingly, all of these regions can be expressed by non-Vγ9Vδ2 T cells responding to CMV, in all possible combinations but with a representation and repartition that can be completely different from one individual to another.[70,78] Thus, all non-Vγ9Vδ2 T cells can be assembled in a single entity able to respond to CMV, as opposed to Vγ9Vδ2 T cells that never respond to the virus. Nevertheless, the Vδ1 region predominates among non-Vγ9Vδ2 T cells in most CMV-infected individuals.[36,78]

Noteworthy, some patients can present an extreme diversity restriction resulting from the major expansion of a subset expressing only one Vγ and Vδ association.[78,104]

The hypervariability of the γδ TCR complementary-determining region 3 (CDR3) accounts for the second level of diversity, called junctional diversity. TCR repertoire junctional diversity of non-Vγ9Vδ2 T cells was initially inferred by measuring CDR3 lengths. This analysis, called spectratyping or immunoscope technique, measures the distribution of CDR3γ and CDR3δ lengths among the sequenced γδ T cells. In KTR,[78] healthy donors,[36] newborns,[70] and HCTR,[83] CMV infection was associated with CDR3 length restriction for the δ1 [36,70,78,83] and δ3 chains.[78,83] This restriction was associated only with CMV infection and not with any of the other herpesvirus infections tested.[36] In KTR, longitudinal monitoring of the repertoire before and after CMV infection showed that infection induced repertoire restriction, suggesting antigen-mediated clonal expansion of specific non-Vγ9Vδ2 T cells.[78] Extreme situations with overrepresentation of one dominating clone, confirmed by CDR3δ sequencing, were observed in some patients.[104] Remarkably, Vermijlen and colleagues described the enrichment of a public germline-encoded Vγ8Vδ1 TCR upon CMV infection in neonates. This is to date the only study to describe the expansion of a public γδTCR in response to CMV.[70] Altogether, these studies show that CMV infection induces the expansion of non-Vγ9Vδ2 T cells with a restricted repertoire, suggesting an antigen-driven clonal selection.

CMV-driven clonal expansion of non-Vγ9Vδ2 T cell was recently formally demonstrated, with the help of next-generation sequencing (NGS), in HTCR.[90] NGS allows a comprehensive analysis of the TCR repertoires with a monitoring of specific CDR3 sequences and clones. Expansion clonality can be confirmed by single-cell sequencing, which allows the association between TRG and TRD amplified sequences. Using these approaches, massive and long-lasting clonal expansions of non-Vγ9Vδ2 T cells were observed only in patients suffering from post-transplant CMV infection.[90] By comparing CMV-positive and CMV-negative bone marrow grafts, another study confirmed the restricted TCR repertoire diversity of clonally expanded non-Vγ9Vδ2 T cells in CMV-seropositive individuals.[89]

Altogether, clonal expansion of non-Vγ9Vδ2 T cell during CMV infection argues for an adaptive and antigen-specific response.

3.3 | Non-Vγ9Vδ2 T cell phenotype associated with CMV-driven expansion

Another evidence for the CMV specificity of non-Vγ9Vδ2 T cells is based on their phenotypic characteristics during the course of CMV infection.

Antigen-driven activation of Vδ2neg T cells following CMV infection was initially described by flow cytometry. Like CMV-specific αβ T cells, Vδ2neg T cells switch from a mainly naive phenotype (CD27+CD45RA+) toward a late-differentiated TEMRA phenotype (CD27-CD45RA+) during the course of CMV infection.[105] Strikingly, CMV seropositivity in healthy individuals is associated with a

complete remodeling of the Vδ2neg compartment from a naive to a late effector phenotype.[36,51,79,106,107] This observation has recently been extended to Vγ9negVδ2pos T cells.[71]

Surface expression of the co-stimulatory receptor CD28 is decreased after TCR activation. When CD28 levels were compared on Vδ2neg T cells from CMV-seropositive versus CMV-seronegative patients,[36] a decreased expression was observed in CMV-seropositive patients, arguing for a TCR engagement on CMV-specific cells. Additionally, Roux and colleagues described an increased expression of the activation marker CD38 during the acute phase of CMV infection, followed by a decrease when CMV became chronic.[108] Finally, we and others [71,108] observed a direct intracellular marker of proliferation (Ki67) on non-Vγ9Vδ2 T cells during the acute phase of CMV infection, which drastically decreased when CMV DNAemia became negative.

Other markers associated with TCR activation are co-inhibitory receptors. Indeed, during T cell antigenic activation, inhibitory receptors such as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and V-domain Ig suppressor of T cell activation (VISTA) are induced and repress immune stimulation, resulting in return to a resting state.[109-111] Recently, we described that PD-1, TIM-3, and LAG-3 levels were increased on non-Vγ9Vδ2 T cells from CMV-seropositive KTR.[71] Additionally, we observed that PD-1 and TIM-3 were highly expressed during the course of both primary infection and reactivation, compared with CMV-seropositive inactive patients, suggesting an antigen-experienced activation of non-Vγ9Vδ2 T cell during CMV infection.[71] Altogether, although CMV is directly recognized by non-Vγ9Vδ2 T cells, there is accumulating evidence in vivo that it drives a somehow specific activation of these cells.

Other phenotypic characteristics have been highlighted on CMV-induced non-Vγ9Vδ2 T cells, showing that they shared many characteristics with CMV-specific CD8+ αβ T cells.[51,54] In CMV-seropositive healthy donors and transplant patients, non-Vγ9Vδ2 T cells are mainly TEMRA cells: CD27−, CD28−, CD45RA+, CD45RO−, CCR7−, CD62L−, perforin+, and granzyme B+ [36,105] with an activated phenotype (CD69+, HLA-DR+).[78]

NK cell receptors (NKRs) for major histocompatibility complex (MHC) class I molecules are important for regulating γδ T cell reactivity.[112] These receptors may belong to either the immunoglobulin or C-type lectin superfamilies and transmit, upon ligand binding, inhibitory or activating signals. CD94/NKG2A and inhibitory killer-cell immunoglobulin-like receptors (KIRs) are inhibitory receptors, whereas CD94/NKG2C, NKG2D, activating KIRs, and natural cytotoxic receptors (NCRs) are activating receptors.[113] CD16 is also a NK receptor recognizing the IgG Fc fragment. Of note, depending on their intracellular domains, KIRs with the same extracellular domains can be either activating (short domain) or inhibitory (long domain).

NK cell receptors can be expressed on both CD8+ CMV-specific T cells and Vδ2neg γδ T cells, but with different patterns. In CD8+ T cells, a rapid and long-lasting change in the expression of NKRs has been

observed during the course of CMV infection.[57] CD158b/j/e, NKG2D, and CD94/NKG2C were upregulated first during infection, and followed by CD94/NKG2A,[57] whereas NKp46 increased very transiently and was not maintained. NKG2D is expressed in CMV-seropositive patients on both T cell compartments, whereas NKG2A is expressed on CD8^{+} but poorly on Vδ2neg γδ T cells, and conversely, CD158 (either b/j or a/h), NKp80, and NKG2C are more expressed on Vδ2neg γδ T cells than on CD8^{+} T cells.[36,105] CD85j (also called ILT2 or LILRB1) is another inhibitory receptor that recognizes UL18, a CMV protein upregulated on infected cell surface, as well as classical and non-classical HLA class I molecules. CD85j expression was reported on Vδ2neg γδ T cells after CMV infection.[104,114] Moreover, CMV-induced Vδ2neg γδ T cells highly express CD16, with an increase during the course of CMV infection in KTR,[115] in contrast to CMV-specific CD8^{+} T cells. Interestingly, CD16^{+} Vδ2neg γδ T cells often co-express NKRs (NKG2D, CD158b/j, and NKp80).[36,115] Despite a clonal expansion after CMV infection in a lung transplant patient, Vδ2neg γδ T cells displayed a heterogeneous NKR phenotype associated with a functional intraclonal diversity.[104] Such diversity could be linked with different regulations of CMV-specific Vδ2neg γδ T cells according to the context, tissue, or infection progression. Moreover, γδ T cells derived from CMV-infected versus CMV-uninfected newborns also showed a similar profile: CD27-, CD28-, HLA-DR[+], NKG2C^{+}, NKG2D^{+}, CD158a/h[+], CD158b/j[+], perforin[+], granzyme A[+], and CX3CR1[+].[70]

Of note, the precise description of inhibitory versus activating KIR expression by flow cytometry is limited by antibody cross-reactivities. For example, in the mentioned studies, the GL183 clone used recognizes CD158j(KIR2DS2)/CD158b1(KIR2DL2)/CD158b2(KIR2DL3), meaning both inhibitory and activating KIRs, whereas the NKb1 clone recognizes CD158e (KIR3DL1/DL2), meaning only inhibitory KIRs.

CD8αα is overexpressed on CMV-induced Vδ2neg γδ T cells from HCTR,[85] newborns,[85] and KTR,[78] and in vivo, its expression has been correlated with CMV infection.

Finally, we recently analyzed the differences between Vδ2neg and Vγ9negVδ2pos T cells, among the non-Vγ9Vδ2 T cell compartment.[71] They were quite similar regarding phenotypic characteristics in CMV-seropositive patients. However, during the course of CMV reactivation, activation markers on Vγ9negVδ2pos T cells were promptly negatively regulated compared with Vδ2neg T cells, and compared with the same markers on both subsets during the course of primary infection.[71]

3.4 | First transcriptomic data on CMV-induced non-Vγ9Vδ2 T cells

More recently, we explored the signature of CMV-induced Vδ1 γδ T cells through single-cell RNA sequencing, in comparison with Vγ9Vδ2 T cells.[116] In this study, 38 genes were differentially expressed between the two populations.[116] These comprised 25 genes, such as KLRC2 (NKG2C), KLRF1 (NKp80), FCGR3A (CD16), GZMB, and GZMH, which were more expressed in TCRVδ1 cells than in TCRVδ2

cells, and 13 genes, including KLRC1 (NKG2A), GZMK, LTB, and IL-7R, which were upregulated in TCRVδ2 cells.[116]

We additionally analyzed, among TCRVδ1 cells, differences between naive versus TEMRA cells in a CMV-seropositive donor. We found 343 differentially regulated genes, including 20 upregulated in naive cells such as IL-7R, CD27, lymphotoxin beta, and NOSIP, and 323 upregulated in TEMRA cells among which perforin, granzyme (B, A, H), CCL4, KLRD1, and KLRF1 showed the highest expression (unpublished personal data).

Altogether, CMV-induced non-Vγ9Vδ2 T cells harbor specific phenotypic markers compared with naive cells, which are classically associated with activating or inhibitory functions. Such markers are of particular interest for a monitoring perspective.

3.5 | Specificity of the non-Vγ9Vδ2 T cell compartment modification during aging

Vδ2neg T cells have also been described in CMV-seropositive elderly individuals, with several studies showing some contradictory results.[98,108,117-120] Of note, the lower limit of age to define old individuals varied from 50-60 years[98,119,120] to 70 years.[108,117,118]

With flow cytometry analysis of peripheral blood comparing old versus young individuals, one study, defining the old age between 50 and 64 years and comparing total γδ T cells, observed that CMV-seropositive old individuals seem to have decreased proportions of γδ T cells compared with CMV-seropositive young individuals.[98] However, when differentiating the Vδ2pos from the Vδ2neg compartment in other studies, this decrease in CMV-seropositive old individuals came from Vδ2pos T cells,[108,120] whereas Vδ2neg γδ T cells seemed to maintain a robust and stable pool through life,[108,117] associated with an increased Vδ1/Vδ2 ratio.[121] Furthermore, Alejenef and colleagues showed that the increased proportion of Vδ2neg γδ T cells in CMV-seropositive versus CMV-seronegative individuals was even more significant in old compared with young individuals.[120] Moreover, phenotypic alterations of the Vδ2neg γδ T cell compartment in the combined contexts of age and CMV have been described. CMV and age were independently associated with high amount of TEMRA Vδ2neg γδ T cells [117,121] to the detriment of the naive compartment. These observations were made in healthy individuals and in lung-transplanted patients and are consistent with CMV as a driving force for the accumulation of late-differentiated γδ T cells, as well described for CD8^{+} αβ T cells.[122,123] Additional studies assessing the phenotypic specificities of TEMRA non-Vγ9Vδ2 T cells in CMV-seropositive old versus young individuals could inform about their degree of senescence and functionality in the elderly.

3.6 | Non-Vγ9Vδ2 T cell anti-CMV reactivity

In vitro, experiments designed to study the reactivity of non-Vγ9Vδ2 T cells against CMV-infected cells provide a last evidence

for their specificity and data regarding the type of functions they develop. Studying non-Vγ9Vδ2 T cell reactivity ex vivo is technically challenging because of the low numbers of these cells in the peripheral blood and the restricted access to human tissues for lymphocyte extraction. In order to assess non-Vγ9Vδ2 T cell responses to CMV-infected cells, peripheral blood mononuclear cells (PBMCs) from CMV-seropositive KTR were initially directly cultured in the presence of CMV lysates,[78] and later with CMV-infected target cells.[71] This method allows the measurement of non-Vγ9Vδ2 T cell proliferation in culture, as well as the quantification of cytokine production by intracellular staining with flow cytometry.[71,78] When working with PBMCs, indirect help signals from other cell populations cannot be deciphered from non-Vγ9Vδ2 T cell responses. Specific responses have to be confirmed using pure cell lines and clones generated in vitro from healthy donor, patient, or newborn PBMCs, using recombinant IL-2, phytohemagglutinin (PHA) and irradiated allogeneic PBMCs.[78,83,124] More recently, we developed an alternative method, more straightforward and less time-consuming, in which Vδ2neg γδ T cell lines are directly expanded from PBMCs of CMV-seropositive KTR with recombinant IL-2 and IL-15.[71]

In order to assess anti-CMV reactivity in vitro, cells lines or clones are cultured with mock-infected or CMV-infected MRC5 cells or primary fibroblasts. Tests are performed either for cytotoxicity [36,70,83,124] by flow cytometry, or for IFN-γ (70,124,125) and TNF-α (83) secretion in supernatants.[126,127] In addition to these assays, the inhibition of CMV replication by Vδ2neg γδ T cell clones has been previously measured by quantifying viral DNA by polymerase chain reaction (PCR) in co-culture supernatants. Viral dissemination assays, which are based on the use of green fluorescent protein (GFP)-expressing viruses, allow the quantification of CMV dissemination in cultures of CMV-infected fibroblasts or myeloid cells. They have been used in order to demonstrate the control of CMV dissemination in vitro by CD4+ T cells,[128] CD8+ T cells,[129] and NK cells.[130] We recently applied this method and observed an inhibition of CMV dissemination by non-Vγ9Vδ2 T cells (personal communication).

Importantly, specific reactivity against CMV-infected cells in vitro was confirmed by co-culturing PBMCs from CMV-seropositive patients with cells infected with the herpesviruses HSV and VZV [71] or with HSV lysates,[78] showing neither proliferation [78] nor intracellular IFN-γ production.[71] Of note, previous studies have focused on fibroblasts as a model to assess non-Vγ9Vδ2 T cell response to CMV. Nevertheless, other cellular models, for instance CMV-infected endothelial cells, epithelial cells, or myeloid cells, could help uncover new properties, and therefore deserve future interest. Moreover, only pro-inflammatory and cytotoxic responses have been studied so far, but other functions could be further tested.

4 | SENSING OF CMV-INFECTED CELLS BY NON-Vγ9Vδ2 T CELLS

Despite their clear role in the anti-CMV immune response, little is known regarding the nature of the antigens recognized by non-Vγ9Vδ2 T cells in this context, as well as the receptors involved. Initial reports showed the importance of the γδTCR in driving the response in vivo [36,70,78,83] and in vitro [70,78,83,120,126] and are now confirmed and completed by high-throughput in vivo γδTCR repertoire analyses.[79,90] However, a growing body of evidence highlights the importance of numerous additional receptors and cues, which also trigger and fine-tune non-Vγ9Vδ2 T cell responses to CMV infection (Figure 2). Here, we review in vivo and in vitro data unveiling the molecular mechanisms of CMV sensing by non-Vγ9Vδ2 T cells.

4.1 | In vitro evidence for the γδTCR importance in non-Vγ9Vδ2 T cell responses against CMV

The in vivo studies described earlier suggest that non-Vγ9Vδ2 T cells respond to CMV after sensing of specific antigens by their TCR. In addition, γδTCR importance in non-Vγ9Vδ2 T cell reactivity against CMV was extensively analyzed in vitro. Indeed, γδTCR-blocking antibodies could abrogate Vδ2neg T cell reactivity against lysates of CMV-infected fibroblasts, demonstrating for the first time the γδTCR importance in response to CMV-induced antigens.[78] This initial observation, made on γδ T cells among PBMCs from CMV-seropositive KTR, was subsequently confirmed using clones derived from CMV-seropositive newborns, or kidney and lung transplant recipients undergoing CMV infection.[70,126,127] Such clones produced IFN-γ or TNF-α and lysed CMV-infected target cells in a TCR-dependent manner. This was confirmed by observing γδTCR downregulation after activation by CMV-infected targets. Moreover, we and others reported, using polyclonal Vδ2neg T cell lines generated in vitro,[83,120] as well as preactivated PBMCs from CMV-seropositive KTR,[71] a decreased response against CMV after TCR-blocking by anti-TCR antibodies. Finally, transferring γδTCRs from certain CMV-reactive clones in TCR-deficient Jurkat cell lines can confer to these cells a reactivity against CMV-infected cells, demonstrating that a CMV-reactive TCR can be sufficient to observe CMV reactivity [131] (and unpublished data).

4.2 | Ligands recognized by γδTCRs in the context of CMV infection

In order to better understand which ligands can be recognized by γδTCRs in the context of CMV infection, it is useful to have an extensive look at γδTCR ligands identified so far. While a recent study described the in vitro generation of MHC-I–restricted γδ T cells specific for melanoma antigens,[132] γδTCRs generally recognize ligands independently of classical MHC-I presentation, in an antibody-like manner. This particularity in comparison with αβ T cells could be involved in γδ T cell response to CMV, which prevents expression of MHC-I-peptide complexes as an escape mechanism from αβTCR recognition (reviewed in [133]). This feature is a major distinction between γδTCRs and αβTCRs. Importantly, comparatively to αβTCRs, very few γδ TCR ligands have been identified so far.
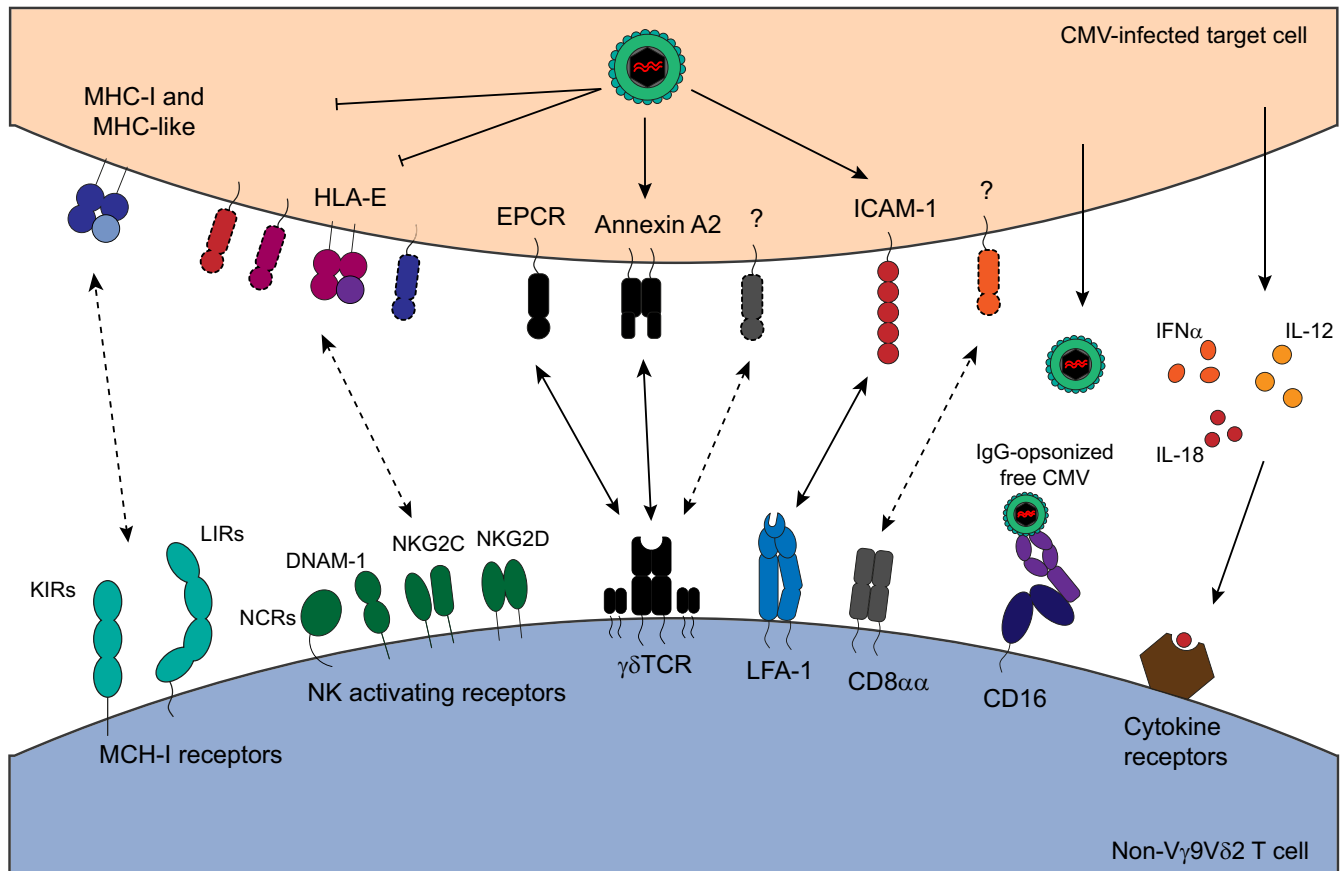
FIGURE 2 Molecular mechanisms underlying cytomegalovirus infection sensing by non-Vγ9Vδ2 T cells. CMV infection induces a multimolecular stress signature, through upregulation of surface molecules such as annexin A2 or intercellular adhesion molecule 1 (ICAM-1), and through the release of pro-inflammatory cytokines such as IFN-α, IL-12, or IL-18. This signature is sensed by non-Vγ9Vδ2 T cells via their T cell receptor (TCR), co-stimulatory receptors such as lymphocyte function–associated antigen-1 (LFA-1), and cytokine receptors. Immunoglobulin G (IgG)-opsonized free CMV can also be recognized by non-Vγ9Vδ2 T cells via CD16. The involvement of other receptors such as major histocompatibility complex I (MHC-I) receptors, natural killer (NK)–activating receptors and CD8αα is suggested but not formally demonstrated. HLA: human leukocyte antigen, EPCR: endothelial protein C receptor, KIRs: killer-cell immunoglobulin-like receptors, LIRs: leukocyte immunoglobulin-like receptors

Vγ9Vδ2 T cells and non-Vγ9Vδ2 T cells harbor distinct properties, including the nature of the ligands they recognize. Sensing of cellular phosphoantigens by Vγ9Vδ2 T cells is mediated by interactions between members of the butyrophilin family and the Vγ9Vδ2 TCR.[134,135] Conversely, ligands identified so far for the TCR of human non-Vγ9Vδ2 T cells are very diverse. They notably include MHC-related proteins such as CD1d,[136,137] CD1c,[138,139] endothelial protein C receptor (EPCR),[127] and major histocompatibility complex class I–related gene protein (MR1).[140] Importantly, their recognition by the γδTCR can be either dependent or independent of antigen presentation by these MHC-like molecules. Non-Vγ9Vδ2 TCR ligands also include diverse membrane-bound or soluble proteins (reviewed in ref. 141). Membrane-bound proteins identified comprise notably annexin A2[131] and ephrin type-A receptor 2 recognized by a Vγ9Vδ1 TCR (our unpublished data). Annexin A2 is an endogenous intracellular protein that displays ectopic expression at the cell surface after oxidative cell stress. Besides, soluble proteins were identified as human γδTCR ligands: histidyl-tRNA synthetase recognized by a human self-reactive Vγ1.3Vδ2 TCR,[142] and phycoerythrin

recognized by subsets of peripheral blood γδ T cells.[143] Altogether, γδTCR ligands identified so far are highly diverse in terms of structure, but present a common feature: They are almost always self-ligands, and their recognition can be induced by cell stress, following transformation or infection. In this review, we will now focus on ligands recognized by the γδTCR of non-Vγ9Vδ2 T cells in the context of CMV infection.

The MHC-like molecules CD1a/b/c/d are not involved in non-Vγ9Vδ2 T cell anti-CMV reactivity,[78,126] and neither is the mucosal-associated invariant T (MAIT) cells and γδ T cell ligand MR1, in which surface expression is inhibited by CMV infection.[144] This last observation raises the question whether CMV has developed immune escape mechanisms targeting other MHC-like molecules potentially sensed by non-Vγ9Vδ2 T cells. To date, a single study has extensively described the recognition of a ligand by a γδTCR in the context of CMV infection.[127] Indeed, the MHC-like protein endothelial protein C receptor (EPCR) was identified as a direct ligand for a Vγ4Vδ5 T cell clone, highly expanded (representing 25% of all blood T cells) in a CMV-infected lung transplant recipient. It

is noteworthy that EPCR surface expression was not modified by CMV infection. Instead, CMV infection triggered a multimolecular stress signature composed of EPCR expression associated with up-regulation of co-stimulatory signals. This stress signature triggered the activation of the Vγ4Vδ5 T cell clone through TCR engagement. Importantly, while the Vγ4Vδ5 clone was the result of a large-scale expansion and dominated the γδTCR repertoire in the individual it was isolated from, it was also private and EPCR reactivity was not observed with other clones. There is currently very little knowledge regarding other ligands recognized in the context of CMV infection. Another identified ligand is annexin A2, in which membrane expression was induced by CMV at the surface of glioblastoma cell lines.[131] Unlike EPCR-induced reactivity, which required a multimolecular stress signature, annexin A2 upregulation at the cell surface was sufficient to trigger the activation of the Vγ8Vδ3 T cell clone used in the study. Annexin A2 is therefore an example of stress ligand induced by CMV and recognized by a γδ TCR and illustrates the concept of stress surveillance by non-Vγ9Vδ2 T cells. Moreover, distinct non-Vγ9Vδ2 T cell clones responded to annexin A2; thus, this ligand likely represents a common ligand to distinct γδTCRs.

The identification of new γδTCR ligands will benefit from recent repertoire analyses, which identified multiple clones drastically expanded in the peripheral blood in response to CMV infection.[90] Besides, identification of ligands recognized by non-Vγ9Vδ2 TCRs within peripheral tissues will require new or adapted strategies but may benefit from recent advances in the isolation and in vitro culture of tissue-resident γδ T cells.[96] For now, identified γδTCR ligands were recognized by a single clone or very little subsets of non-Vγ9Vδ2 T cells, which limits their physiological relevance. The extent of ligands potentially recognized by all the different clones expanded in CMV-infected patients could be huge. The repertoire of Vδ1 TCRs has been shown to be highly private even in CMV-infected individuals,[79] raising the question whether it exists a particular CDR3-independent superantigenic mode of activation of CMV-responding non-Vγ9Vδ2 T cells, or a myriad of antigens related to CMV infection.

Butyrophilins are now extensively described as mediators of phosphoantigens sensing by Vγ9Vδ2 T cells, through their interaction with the Vγ9Vδ2 TCR.[134,135] However, recent studies have also demonstrated butyrophilin bindings by non-Vγ9Vδ2 TCRs, more precisely a Vγ4Vδ5 TCR [145] and a Vγ9Vδ1 TCR.[134] However, the importance of butyrophilins in CMV sensing by this particular TCR was not addressed. More generally, the impact of CMV infection on butyrophilins expression and their binding to γδ TCRs is currently unknown.

As previously mentioned with the Vγ4Vδ5 clone,[127] the reactivity of non-Vγ9Vδ2 T cells against CMV may also be tuned by additional activating and inhibitory receptors, which may act independently of the TCR or as co-stimulatory signals (Figure 2). We will now focus on such receptors and their involvement in CMV sensing.

4.3 | CD16

As described above, Vδ2neg γδ T cells amplified in the peripheral blood of CMV-seropositive patients and healthy individuals express the Fc receptor CD16 (FcRγIIIa), which interacts with the Fc portion of IgG.[124] This observation suggests a role for CD16 in non-Vγ9Vδ2 T cell anti-CMV immune response. It is now well described that NK cells can respond to CMV-specific antibodies via CD16, by mediating antibody-dependent cell cytotoxicity (ADCC) and by producing antiviral cytokines such as IFN-γ and TNF-α in vitro.[47,146,147] Vδ2neg γδ T cells however do not appear to mediate ADCC against CMV-infected cells. Instead, CD16+ Vδ2neg γδ T cell lines can produce IFN-γ in response to IgG-opsonized CMV virions. In line with its described antiviral properties, IFN-γ production by Vδ2neg γδ T cells is sufficient to inhibit CMV replication in culture. CD16 is therefore proposed as a mediator of antibody-dependent cell-mediated inhibition (ADCI) of CMV replication by Vδ2neg γδ T cells.[124] Importantly, CD16 does not function as a co-receptor in this context, meaning that CD16 engagement is sufficient to mediate Vδ2neg γδ T cell activation, independently of the TCR. In vivo, CMV-induced Vδ2neg γδ T cells expressing CD16, present in large numbers in the peripheral blood and homing to tissues, could therefore play an important role in targeting opsonized CMV virions without the need of antigen recognition by the TCR. Moreover, as CMV-specific CD8+ T cells do not express CD16,[124] ADCI would represent a mechanism of control of CMV infection specific to NK cells and γδ T cells, broadening the arsenal of anti-CMV immune functions.

4.4 | LFA-1 co-stimulation

Lymphocyte function–associated antigen-1 (LFA-1 or CD11a-CD18) is a member of the integrin family, and a regulator of T cell functions such as migration, adhesion, and activation. It is notably involved in their interaction with endothelial cells, antigen-presenting cells (APCs), and their binding and subsequent killing of target cells (reviewed in ref. 148). Its ligand intercellular adhesion molecule 1 (ICAM-1 or CD54) is expressed on a large panel of cell types such as activated endothelial cells, fibroblasts, myeloid cells, B cells, and T cells. ICAM-1 is notably overexpressed on CMV-infected macrophages,[149] fibroblasts,[127,150] and endothelial cells.[127,151] However, other integrins are downregulated on CMV-infected fibroblasts [152] and macrophages,[149] suggesting a complex regulation of integrin expression by CMV infection. The implication of the LFA-1-ICAM-1 axis in the response against CMV has been described for CD8+ and CD4+ T cells [153,154] as well as NK cells [155] in vitro. Regarding non-Vγ9Vδ2 T cells, LFA-1-mediated co-stimulation was described in the context of EPCR binding to a Vγ4Vδ5 TCR.[127] EPCR was not upregulated after CMV infection, but constitutively expressed at the cell membrane, and its expression was not sufficient to trigger recognition of CMV-infected targets. However, CMV-induced ICAM-1 expression played a significant role in the activation of the Vγ4Vδ5 clone. Altogether, ICAM-1 upregulation represents

an interesting example of stress signature specific to CMV infection, which can be sensed by non-Vγ9Vδ2 T cells.

4.5 | CD8αα co-stimulation

CD8αα is expressed on NK cells,[156] αβ T cells[157] and Vδ2neg γδ T cells[158,159] and is frequently associated with intraepithelial lymphocytes.[157] While CD8αβ heterodimers are well identified as a co-receptor required for MHC-I–restricted ligand recognition by the αβTCR, CD8αα function was initially not characterized. Interestingly, CD8αα is overexpressed on CMV-induced Vδ2neg γδ T cells from HCTR,[85] newborns,[85] and KTR.[78] CD8αα was described as a co-receptor involved in the recognition of leukemic cells by Vδ2neg γδ T cell clones isolated from HCTR undergoing CMV infection.[85,160] However, its implication in the recognition of CMV-infected cell was unclear, because CD8αα decreased not only reactivity toward CMV-infected cells but also the basal reactivity toward uninfected cells.[85] CD8αα co-stimulation in the context of CMV therefore deserves future investigation, as does the identification of the ligand recognized by CD8αα in this context.

4.6 | NK-activating receptors and MHC-I receptors

Several NK-activating receptors may be expressed by non-Vγ9Vδ2 T cells in physiological contexts or after in vitro stimulation, such as NKG2D, NKG2C, DNAX accessory molecule 1 (DNAM-1), 2B4, and natural cytotoxicity receptors (NKp30, NKp44, NKp46).[161-163]

Such receptors were shown to be involved in the recognition and killing of cancer cells and the control of HIV infection, by NK cells or Vγ9Vδ2 T cells.[162,164-166] Nevertheless, the reactivity of non-Vγ9Vδ2 T cell clones against CMV is independent of NKG2D[126] and the involvement of other NK-activating receptors in anti-CMV non-Vγ9Vδ2 T cell response remains to be investigated. Of note, as CMV has developed multiple evasion strategies to escape from NK cell recognition through these activating receptors,[167-171172] one can speculate that this escape also takes place with non-Vγ9Vδ2 T cells.

CMV has also developed multiple immune evasion strategies preventing HLA-I expression at the cell surface and allowing escape to CD8+ T cell responses. As NK cells are able to sense the absence of HLA-I through inhibitory receptors (sensing of "missing-self"), such receptors are involved in their response against CMV infection.[14] Interestingly, as previously said, CMV-induced non-Vγ9Vδ2 γδ T cells express higher levels of various KIRs,[36,70,105] and of the inhibitory receptor CD85j,[114] compared to Vδ2neg γδ T cells from CMV-seronegative individuals. These observations suggest a potential role for these receptors in modulating non-Vγ9Vδ2 γδ T cell responses against CMV. Besides, an in vitro study described the expression of KIRs and CD85j on a Vγ4Vδ5 γδ T cell clone isolated from a lung transplant recipient.[104] Such receptors influenced the function of the clone, as KIR2DS2 had a co-stimulatory effect on IFN-γ and TNF-α secretion after TCR engagement. However, the importance of these MHC-I receptors in the response against CMV-infected cells was not evaluated.

4.7 | Importance of the cytokine environment in CMV sensing by non-Vγ9Vδ2 T cells

In addition to different receptors recognizing CMV-induced antigens or missing-self, the cytokine environment tunes γδ T cell activation and effector functions in different physiological and pathogenic contexts.[166] Here, we focus on cytokines induced by CMV infection, with identified role in the regulation of non-Vγ9Vδ2 γδ T cell responses.

During the course of CMV primary infection and subsequent reactivation events, myeloid cells such as monocytes, macrophages and DCs play a central role as CMV targets and antigen-presenting cells. They are known producers of pro-inflammatory cytokines such as type I IFNs and IL-12. IFN-α and IL-12 are upregulated at the transcriptional and protein levels and secreted by CMV-infected macrophages.[149,173,174] In addition, CMV-infected DCs produce IFN-α, IFN-β, IFN-λ, and IL-12.[175] IFN-α and IL-12 enhance CD16-induced IFN-γ production by Vδ2neg γδ T cells and subsequent control of CMV replication in vitro.[124] Of note, IL-12 positively regulates IFN-γ production by NK cells in response to CMV-infected macrophages[173] underlying the pleiotropic effect of the cytokine environment on different immune effectors. Similarly, secretion of the pro-inflammatory cytokine IL-18 is induced on CMV-infected endothelial cells in vitro, through caspase-1 inflammasome activation.[125] Its expression is also induced at the transcriptional level on CMV-infected macrophages.[149] When Vδ2neg γδ T cells are activated in vitro through their TCR, they express the high-affinity IL-18 receptor IL-18Rβ at their membrane and become more sensible to IL-18. They sense IL-18 induced by CMV infection, and IL-18 potentiates their IFN-γ production induced by TCR stimulation. Ultimately, IL-18 tunes Vδ2neg γδ T cell response to CMV-infected targets in a TCR-dependent manner.[125]

These findings regarding IL-18 illustrate how TCR engagement can set-up non-Vγ9Vδ2 T cells for the sensing of signals, for instance small inflammatory molecules, enhancing non-Vγ9Vδ2 T cell tissue stress surveillance. Besides, because a synergy between TCR or CD16 engagement and cytokine signaling is essential for an optimal response, non-Vγ9Vδ2 T cell activation in vivo would be restricted to a well-restricted environment induced by CMV infection. These several layers of control seem capital, especially for cells recognizing self-ligands.

We presented above γδ T cell features and functions in the context of CMV infection, as well as the molecular mechanisms involved in CMV sensing. We will now discuss how these features can be harnessed in different clinical settings, and especially in the field of transplantations.

5 | HARNESSING NON-Vγ9Vδ2 T CELLS FOR ADOPTIVE CELL THERAPY

HCT requires conditioning therapy and post-transplant immunosuppression, resulting in a highly impaired immune system, which slowly reconstitutes. Complications post-HCT notably include

opportunistic viral infections. Despite efficient preemptive strategies and early detection, CMV infection and subsequent disease remain lethal. In SOTR, CMV infection and disease are still associated with important complications, notably an increased risk of graft rejection, as well as significant mortality and morbidity, despite prophylaxis or preemptive therapies. In patients undergoing CMV disease that are resistant to antiviral treatments, second-line therapies are associated with important adverse effects, and no other therapeutic option is currently available.[5] Importantly, it has been shown that a quicker immune reconstitution of both αβ and γδ T cell compartments after HCT or a better quality of the αβ and γδ T cell responses in SOTR is correlated with a lower incidence of CMV infection and disease.[34,63,176-178] These observations highlight the importance of an efficient cellular immune response for preventing CMV infection.

Adoptive transfer of CMV-reactive T cells therefore represents an attractive approach in both HCTR and SOTR, in order to boost the cellular immune response and achieve long-term protection against CMV infection. So far, adoptive transfer of CMV-specific αβ T cells in HCTR has been the most described, and first studies are starting to report their use in SOTR. However, a cell therapy strategy using γδ T cells remains to be explored. Here, we detail the state of the art regarding αβ T cell therapies for CMV infection in HCTR and SOTR, as well as their current limitations and the requirements for a successful adoptive therapy. Next, taking into account the recent development of preclinical protocols of non-Vγ9Vδ2 T cell adoptive therapy for cancer treatment, we argue that a non-Vγ9Vδ2 T cell therapy for the treatment of CMV infection in transplant recipients holds great potential and should be explored.

5.1 | State of the art and limitations of cell therapies for the treatment of CMV infection in transplant recipients

5.1.1 | In vitro expansion or direct isolation of CMV-specific αβ T cells for adoptive transfer in HCTR

Several good manufacturing practice (GMP)-compatible strategies have been developed in order to either directly isolate or expand in vitro CMV-specific αβ T cells for adoptive transfer in HCTR (for recent reviews, see ref. 177, 179). Briefly, a first strategy consists in expanding polyclonal CMV-specific CD4+ and CD8+ T cells in vitro, using co-cultures with autologous DCs or artificial APCs, loaded with overlapping CMV peptide pools. The feasibility and safety of this method was shown in phase I and II clinical trials of adoptive transfer.[177] Its large-scale clinical application is however hindered by extensive in vitro culture and by the heterogeneity of cellular products. In order to limit in vitro manipulations, methods allowing the direct isolation of CMV-specific αβ T cells have been developed: (i) the use of HLA multimers loaded with HLA-restricted viral peptides, allowing the magnetic sorting of HLA-restricted CD8+ T cells or (ii) IFN-γ capture assays allowing the magnetic sorting of αβ T

cells secreting IFN-γ in response to a CMV-specific stimulation. The second method has the advantage of isolating both CD4+ and CD8+ T cells, which are not restricted by a specific HLA. Direct isolation strategies have been used in phase I and II clinical trials in HCTR, with promising clinical responses.[177,179]

5.1.2 | αβ T cell adoptive therapy in solid organ transplant recipients

While adoptive T cell therapy for HCTR has already been extensively described in clinical trials, its application in the context of solid organ transplant (SOT) has been very poorly explored. This gap can be explained by several complications specific to solid organ transplantation: (a) Autologous material used to generate CMV-specific αβ T cells for adoptive therapy in HCTR comes from the healthy stem cell donor, whereas autologous therapy in the SOT setting requires the manufacture of CMV-specific αβ T cells from an immunosuppressed patient, with functionally impaired T cells. (b) In HCTR, cell therapy is infused at a time when the endogenous T cell compartment is not fully formed. This favors the homeostatic proliferation of transferred cells. Conversely, transferred cells in a SOT setting would compete with endogenous T cells for proliferation and long-term survival. (c) After transfer, the strong immunosuppressive regimen given to SOTR can inhibit the response of infused cells.

αβ T cell adoptive therapy was initially proposed as salvage therapy in four case reports of three lung transplant recipients[180-182] and one kidney transplant recipient[183] all undergoing life-threatening, antiviral-resistant CMV infection or disease. The first case report in a lung transplant recipient showed limits for feasibility and efficacy and concerns regarding safety.[180] However, two subsequent case reports showed the successful autologous expansion of polyfunctional and multispecific αβ T cells in clinically relevant numbers. αβ T cells were obtained in vitro after co-culture with autologous irradiated PBMCs loaded with CMV peptides. In these two reports, T cell infusion was associated with no adverse effects. Transferred cells persisted after infusion and CMV infection was controlled in the long-term in the two lung transplant recipients.[181,182] Besides, in a KTR, αβ T cells from a partially HLA-matched third-party donor were expanded in vitro using autologous monocyte-derived DCs pulsed with CMV peptides, and subsequently infused with only low-grade adverse effects. The case report suggested protection against CMV infection, but lacked an immunological monitoring of infused T cells.[183]

After these promising reports, a recent phase I clinical trial described for the first time the use of autologous αβ T cell adoptive therapy in SOTR. Smith and colleagues treated 22 SOTR (kidney, lung, heart) undergoing recurrent or resistant CMV infection or disease, with autologous CMV-specific αβ T cells amplified in vitro. Cell therapy manufacture relied on PBMC stimulation with an overlapping CMV peptide pool targeting several immunodominant antigens. Importantly, manufacture was successful and generated clinically relevant cell numbers in 20 out of 21 patients, demonstrating the clear feasibility of this approach. Patients received between two and

six T cell infusions, with a median of 1.92×10^8 transferred cells in total per patient.[184] Amplified cells were polyfunctional and specific to multiple CMV epitopes. Adoptive transfer was not associated with serious adverse effects and had no effect on graft survival, demonstrating safety. Finally, although this trial was not designed to assess efficacy, 11 patients out of 13 showed symptom improvement after transfer.[185] Of note, infused cells were monitored for persistence, differentiation, and functionality, as well as for the evolution of their TCR repertoire.[184,185] CMV-specific αβ T cells persisted in several patients maintained polyfunctionality and an effector phenotype.

5.1.3 | What are the limits associated with CMV-specific αβ T cell manufacture and adoptive transfer in HCTR and SOTR?

Despite an accumulating number of clinical trials, several limits hinder the large-scale use of αβ T cell adoptive therapy in HCTR. First, for a majority of strategies, αβ T cell manufacture generates αβ T cells restricted to a specific HLA and to a few CMV epitopes, used for stimulation. Second, in vitro extensive culture or ex vivo isolation select a majority of cells with a late effector phenotype (CD27neg), which have poor proliferative capacities.[177,186,187] Poor multispecificity for CMV peptides and late differentiation negatively impact αβ T cell persistence and efficacy after transfer.[184,188] Third, all the strategies described above have been developed using CMV-seropositive HCT donors. Direct isolation of CMV-specific αβ T cells from CMV-naive donors is technically nearly impossible, and in vitro manufacture of de novo CMV-reactive T cells is not achieved by standard protocols. αβ T cell adoptive therapy is therefore restricted to HCTR receiving a graft from a CMV-seropositive donor. This limitation can be overcome by using third-party CMV-seropositive donors, but in this case, partial HLA mismatch increases the risk of graft-versus-host disease (GVHD) after transfer.[189] The use of third-party donors remains to be assessed in large-scale clinical trials. Alternatively, few studies have recently described the in vitro manufacture and characterization of CMV-reactive αβ T cells from CMV-seronegative cord blood and adult donors[190-192] and protection was suggested in vivo.[191] It however remains unclear whether these methods can generate sufficient cell numbers for clinical use. Importantly, a recent clinical trial described for the first time the expansion of CMV-reactive cells from an initial CMV-naive T cell pool.[184,185] This success in immunosuppressed SOTR, if confirmed in healthy CMV-seronegative donors, could be used to propose adoptive cell therapy in HCT settings with a CMV-seronegative donor. Finally, in the SOT context, another limitation of αβ T cell therapies linked to their HLA restriction deserves attention. Several studies in KTR indeed suggest that when a CMV-seropositive recipient receives a kidney allograft from a CMV-seropositive and HLA class I incompatible donor, CMV-infected cells from the graft or the donor would not be recognized by the recipient's CMV-specific $CD8^+$ T cells.[193-195] This limit could hinder the efficacy of autologous adoptive αβ T cell therapy in D^+R^+ KTR.

Altogether, αβ T cell adoptive therapy holds great promises for the treatment of recurrent or resistant CMV infection and disease in the transplantation setting. However, in HCTR, it is currently limited by the scope of eligible patients, and by issues inherent to αβ T cell HLA restriction, alloreactivity, and late differentiation. In SOTR, recent important progress has been made, but clinical data remain scarce.

5.1.4 | Requirements for a successful adoptive therapy in the context of CMV infection

Previous clinical trials outline several aspects, which seem essential for the success of an anti-CMV cell therapy. These key factors should be thoroughly assessed in future studies (Figure 3).

Regarding feasibility, cell therapy should be achievable at a large scale and minimize costly and time-consuming in vitro manipulations. The cellular product obtained should be reproducible and homogeneous between donors with a high purity for the cell population of interest. Besides, a maximum of patients should be eligible for the therapy. A standardized off-the-shelf therapy seems to be the most appropriate strategy to fulfill these requirements.

The safety and tolerability of the adoptive therapy should be carefully assessed. Preclinical murine models may help estimate safety but are not available for human CMV infection. An alternative approach might be the ex vivo assessment of off-target effects against a panel of primary cells from healthy tissues or organoids. But altogether, safety can be truly evaluated with phase I clinical trials.

Regarding efficacy, three characteristics of the cellular product are essential: polyfunctionality, multispecificity against several CMV epitopes, and fitness, with low differentiation and high proliferative capacity. The importance of persistence after transfer is well illustrated by clinical trials of αβ T cell adoptive transfers in HCTR[196-198] and SOTR.[181,184,185] In particular, transfer of naive and central memory αβ T cells is correlated with better responses, compared with terminally differentiated effector memory cells.[180,184,188]

5.2 | State of the art of non-Vγ9Vδ2 T cell–based therapies against cancer

Until very recently, using CMV-reactive non-Vγ9Vδ2 T cells as a cell therapy would have been hampered by their scarcity in the peripheral blood and the lack of in vitro expansion protocols. Nevertheless, non-Vγ9Vδ2 T cells are now being developed as a cell therapy against multiple cancers, opening new perspectives for their use as an anti-CMV cell therapy.

Initially, several studies described the in vitro expansion of non-Vγ9Vδ2 T cells by using T cell mitogens, namely the plant lectins concanavalin A (Con A) or PHA[199-203] but also the anti-CD3 antibody clone OKT-3,[204,205] in combination with γ_c cytokines such as IL-2, IL-7, IL-15, or IL-21. γ_c cytokines are essential for T cell survival, proliferation, and activation. These expansion methods are based on initial γδ T cell sorting followed by at least two to three weeks of culture,
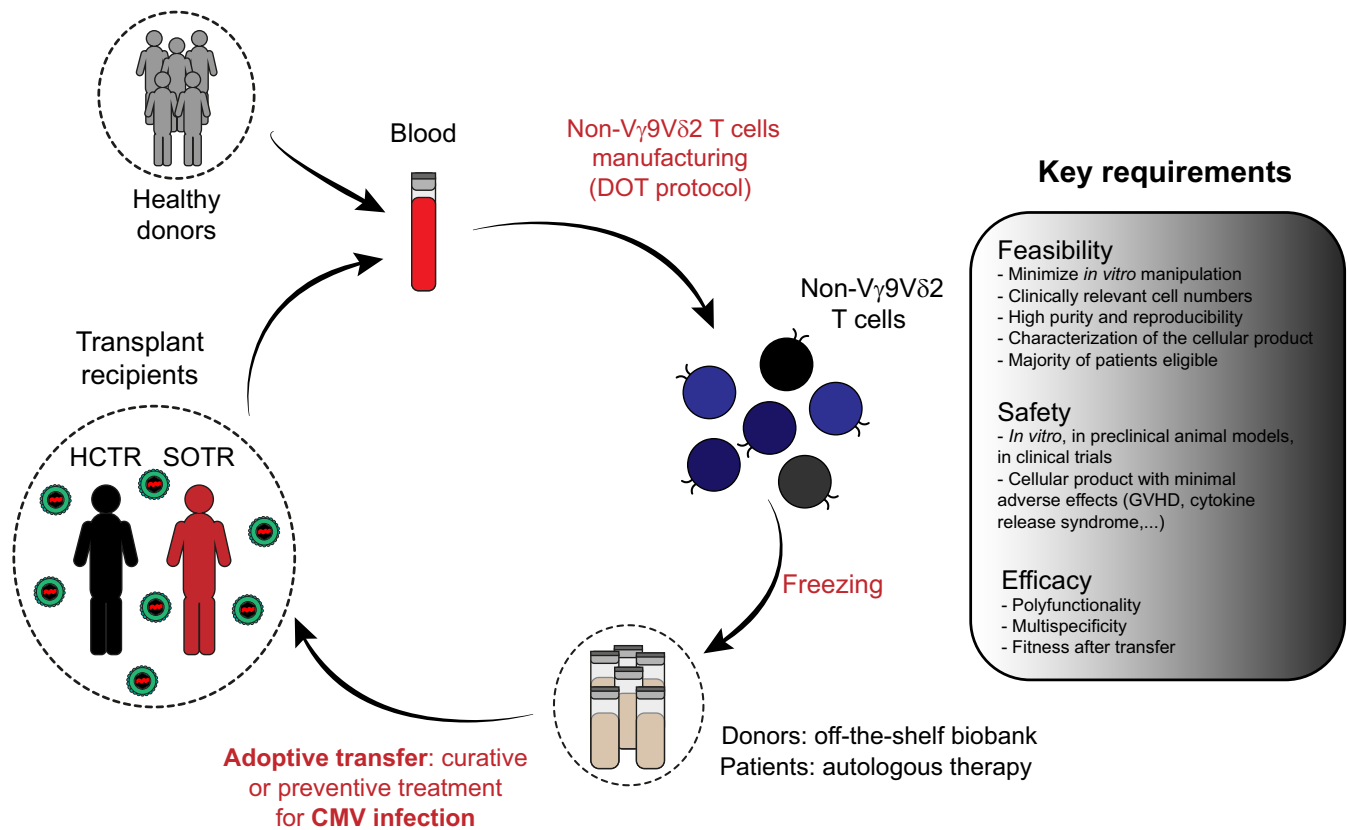
FIGURE 3 Non-Vγ9Vδ2 T cell adoptive transfer strategy for the treatment or prevention of cytomegalovirus infection and disease in transplant recipients. Autologous non-Vγ9Vδ2 T cells are amplified from HCTR or SOTR and frozen before infusion. Alternatively, non-Vγ9Vδ2 T cells can be amplified from healthy donors to constitute a frozen biobank and allow an off-the-shelf therapy. Cell therapy success relies on an optimized cell manufacture protocol, a thorough safety evaluation, and an immunological monitoring to assess functionality and fitness after transfer. HCTR: hematopoietic cell transplant recipient, SOTR: solid organ transplant recipient, DOT: Delta One T, GVHD: graft-versus-host disease

and rely on the proliferation advantage of non-Vγ9Vδ2 T cells after mitogen stimulation, as they are less prone to activation-induced cell death (AICD). After in vitro expansion, a high majority of Vδ1 T cells is obtained. Altogether, these reports establish a preclinical rationale for cell therapy, as they demonstrate non-Vγ9Vδ2 T cell expansion from cancer patients, with reactivity against various solid and hematological malignancies. They are, however, not directly adaptable to clinical use, because they lack clinical-grade reagents for expansion. Two important preclinical studies relied on artificial APCs as feeders, in combination with the γ_c cytokines IL-2 and IL-21.[206,207] In both studies, a polyclonal mix of Vδ1[+], Vδ2[+], and Vδ1negVδ2neg T cells was obtained after expansion. Importantly, Deniger and colleagues described a broad reactivity against different cancers, providing a rationale for a therapy transferable to large numbers of patients. Eliminating the use of mitogens such as PHA or Con A is important for clinical translation, but these strategies still require extensive and time-consuming in vitro manufacture 2-5 weeks with genetically modified and irradiated feeders. Moreover, they generate heterogeneous cellular products between donors in terms of purity, which is not optimal for a large-scale clinical use.

More recently, Almeida and colleagues[161] described an expansion protocol specific of Vδ1 T cells, based on γδ T cell isolation followed by 21 days of culture with OKT-3 and the γ_c cytokines IL-4, IL-15 and IL-21, as well as IL-1β and IFN-γ. This protocol generates a cellular product composed in high majority of Vδ1 cells, but also of other non-Vγ9Vδ2 populations, notably Vδ3 cells. They assessed the preclinical potential of these amplified Delta One T (DOT) cells, showing in vitro reactivity, tumor growth inhibition and survival improvement in xenograft models of both chronic lymphocytic and acute myeloid leukemia. The DOT protocol was designed in order to induce the expression of NK receptors, in particular NCRs (NKp30 and NKp44), which were involved in cancer cell killing, here B-CLL cell lines and primary B-CLL cells from patients.[161] Finally, the expansion protocol induced the expansion of naive CD27^{+} cells and generated a highly polyclonal cellular product.[208] The DOT cell protocol currently seems to be the most adapted to future clinical use, for the following reasons: (a) It expands large-scale numbers of non-Vγ9Vδ2 T cells in a highly specific and reproducible manner, from both healthy donors and cancer patients. (b) It does not require feeder cells, uses clinical-grade reagents, and is compatible with clinical-grade cell culture bags, facilitating transition to production in GMP conditions. (c) The phenotype of manufactured cells has been extensively characterized, notably for markers of activation, differentiation, checkpoint inhibitors, homing and migration receptors, and co-stimulatory

receptors. (d) Safety was characterized in vitro by co-culture with multiple autologous lymphoid and myeloid cells. (e) Mice xenograft preclinical models demonstrated the in vivo persistence and proliferation of transferred DOT cells and also showed efficacy.

Altogether, these preclinical studies have now strongly established the feasibility of adoptive therapies based of non-Vγ9Vδ2 T cells, and in particular Vδ1 and Vδ3 T cells, to treat various cancers. However, such models do not allow a complete safety assessment, which has to be demonstrated in phase I clinical trials.

5.3 | Rationale for a non-Vγ9Vδ2 T cell–based cell therapy for the treatment of CMV infection in transplant recipients

Clinical data show the importance of both αβ T cells and non-Vγ9Vδ2 T cells in vivo in SOTR and HCTR undergoing CMV infection and disease. So far, only αβ T cell therapy has been explored in this context. However, as described above, protocols for non-Vγ9Vδ2 T cell expansion compatible with clinical use are now available for cancer treatments, and the cross-reactivity of non-Vγ9Vδ2 T cells against cancer and CMV has been strongly established in vitro.[85,126,127]

In vitro amplified non-Vγ9Vδ2 T cells therefore represent a relevant alternative for adoptive therapy against CMV infection in transplant recipients: (a) Non-Vγ9Vδ2 T cells are mainly tissue-resident, where they are involved in lymphoid stress surveillance without requirement for prior antigen priming and presentation by APCs. This is associated with a faster response compared with αβ T cells and also implies that non-Vγ9Vδ2 T cells responses are not impacted by CMV immune escape mechanisms targeting APCs. (b) As γδ T cell TCR recognition is not HLA-restricted, non-Vγ9Vδ2 TCRs are not targeted by CMV immune escape mechanisms inhibiting MHC presentation, unlike αβ TCRs. (c) Absence of HLA restriction is also particularly relevant for allogeneic therapies. While autologous αβ T cell therapies are the gold standard, notably because of issues of HLA incompatibility, a non-Vγ9Vδ2 T cell therapy could be developed at a large scale using third-party donors. This off-the-shelf strategy would allow prior validation and cryopreservation of a biobank rapidly available for transfer in patients (Figure 3). (d) Another promising aspect is the shared reactivity of non-Vγ9Vδ2 T cells against CMV and different cancers.[85,126,127] HCTR and SOTR are at risk of leukemia relapse and solid cancers, respectively.[209] In KTR, CMV infection induces Vδ2neg γδ T cell expansion, which is correlated with protection against cancer development.[210] This suggests that CMV-induced Vδ2neg γδ T cells also recognize cancer cells in vivo. This shared reactivity could be harnessed in order to target with a single-cell therapy both CMV infection and cancer in these immunocompromised patients particularly exposed to both CMV and cancer. (e) So far, cancer clinical trials using Vγ9Vδ2 T cell therapies showed a good safety profile with little evidence of side effects such as cytokine release syndrome (reviewed in ref. 207). (f) Vδ2neg γδ T cells are less prone to AICD, in comparison with Vγ9Vδ2 T cells for instance.[211,212]

Despite these promising aspects, some important questions need to be answered before testing a non-Vγ9Vδ2 T cell therapy in CMV-infected transplant recipients: In particular, is non-Vγ9Vδ2 T cells amplification with the DOT protocol similar between CMV-seropositive and CMV-seronegative donors or patients, and can it be achieved from SOTR receiving strong immunosuppression? Besides, are the DOT cells reactive against CMV in vitro? Finally, a clear understanding of the molecular mechanisms underlying CMV-infected cell recognition by DOT cells would be crucial. When these major points will be elucidated, autologous or off-the-shelf non-Vγ9Vδ2 T cell therapy could become a potential new CMV therapy for resistant or recurrent CMV infection and disease, but could also be proposed as preventive therapy in order to avoid the frequent side effects caused by current antiviral drugs (Figure 3).

6 | HARNESSING NON-Vγ9Vδ2 T CELLS FOR IMMUNOMONITORING IN TRANSPLANT RECIPIENTS

6.1 | State of the art of CMV immunomonitoring

As described above, the establishment of a robust and specific adaptive immune response prevents the occurrence of symptomatic CMV events in immunocompetent hosts. However, in immunocompromised hosts who lack such a response, the virus becomes highly replicative and leads to CMV disease with direct organ injuries.[2] Antiviral treatment is necessary until an adaptive immune response is (re)established. However, antiviral treatment strategies can be associated with toxicity, with notably myelotoxicity ((val)ganciclovir) or nephrotoxicity (foscavir). For now, risk stratification of CMV infection and disease relies on CMV serology for both donor (D) and recipient (R), with the highest risk for D⁺R⁻ patients in solid organ transplant patients and for D⁻ patients in HCTR. Unfortunately, despite preventive strategy based on this risk stratification, CMV disease occurs in 5%-12% of R⁺ patients and in 20% of D⁺R⁻ patients.[3,4,115,213,214] Curative antiviral treatment until CMV DNAemia negativation is currently recommended,[5] but viral and clinical relapse occur respectively in 30% and 15% of patients,[215] indicating that curative treatment duration could be improved. Decreasing immunosuppressive treatment has been recommended during severe forms with high viral load in order to facilitate viral clearance,[5,216] but this strategy has been poorly evaluated so far, and studies do not provide clues regarding when and for which patients it could be beneficial. For all those situations, the monitoring of CMV-specific αβ T cell–mediated immunity (CMI) has been evaluated in SOTR and HCTR in order to avoid excessive antiviral treatment and adjust its duration to minimize side effects. This immunomonitoring can be applied to four clinical scenarios in solid organ and hematopoietic cell transplantation (Figure 4).

Commercially available assays monitoring CMV-specific αβ T cell response are the QuantiFERON-CMV assay (Quiagen, USA), as well as ELISPOT CMV assays with T-Track CMV (Lophius Biosciences,

Germany) and T-SPOT CMV (Oxford Immunotec, UK). Results are given after 30-40 hours and inform on both $CD4^+$ and $CD8^+$ responses after activation with IE1 and/or pp65 overlapping peptides. Positive thresholds are necessary to strongly assess the presence of a robust response. For QuantiFERON-CMV assay, the threshold is comprised between 0.1 and 0.2 IU/mL depending on the study.[33,217] The threshold for ELISPOT is more controversial, but has been recently evaluated as predictive only for IE1 at 20 IFN-γ spots/3.10^5 PBMC (Immunotec),[63,218] by Jarque and colleagues, and for pp-65 or IE1 at 40 IFN-γ spots/3.10^5 PBMC[219] (Immunotec).

6.2 | CMV-specific αβ T cell monitoring in solid organ transplant recipients

6.2.1 | At the beginning of transplantation to predict the risk of CMV infection or disease

In D^+R^- patients, T-SPOT > 10 IFN-γ spots/3.10^5 PBMC was found in 30% of patients and was associated with a lower incidence of CMV infection, demonstrating that seronegative recipients could have a protective CMV-specific αβ T CMI.[220] In R^+ patients, at day 0 and more predictively at day 15 post-transplantation, T-SPOT > 20 IFN-γ spots/3.10^5 PBMC for IE1 was highly associated with the absence of any CMV infection in patients who did not receive T-cell–depleting agents as induction therapy.[63] Indeed, we have previously shown that R^+ patients with positive IE1 T-SPOT at day 0 of transplantation,

who received anti-thymocyte globulins, underwent a drastic decrease in IE1 T-SPOT after transplantation for 3 months, and then were at higher risk of developing CMV infection.[221]

6.2.2 | During antiviral prophylaxis to determine for how long it is needed

After antiviral prophylaxis discontinuation, positive T-SPOT[218,219] and positive QuantiFERON-CMV[217] are associated with very low risk of CMV DNAemia and disease.

Consequently, one interventional study using T-Track within the first month post-transplantation (Clinicaltrial.gov, last post-April 2018, Manuel NCT02538172) is ongoing to determine whether this test could be useful for determining the duration of CMV prophylaxis.

6.2.3 | At the beginning of CMV infection in asymptomatic patients

Using QuantiFERON-CMV (threshold > 0.2 IU/mL) at the onset of asymptomatic low-level CMV DNAemia, Lisboa and colleagues observed a positive QuantiFERON-CMV in 92.3% of patients with spontaneous CMV clearance, who did not receive any curative treatment.[222] One interventional study using T-SPOT at the beginning of infection (Clinicaltrial.gov, last post-October 2018, Bestard NCT02550639) is ongoing. If these retrospective results are confirmed, it would allow to
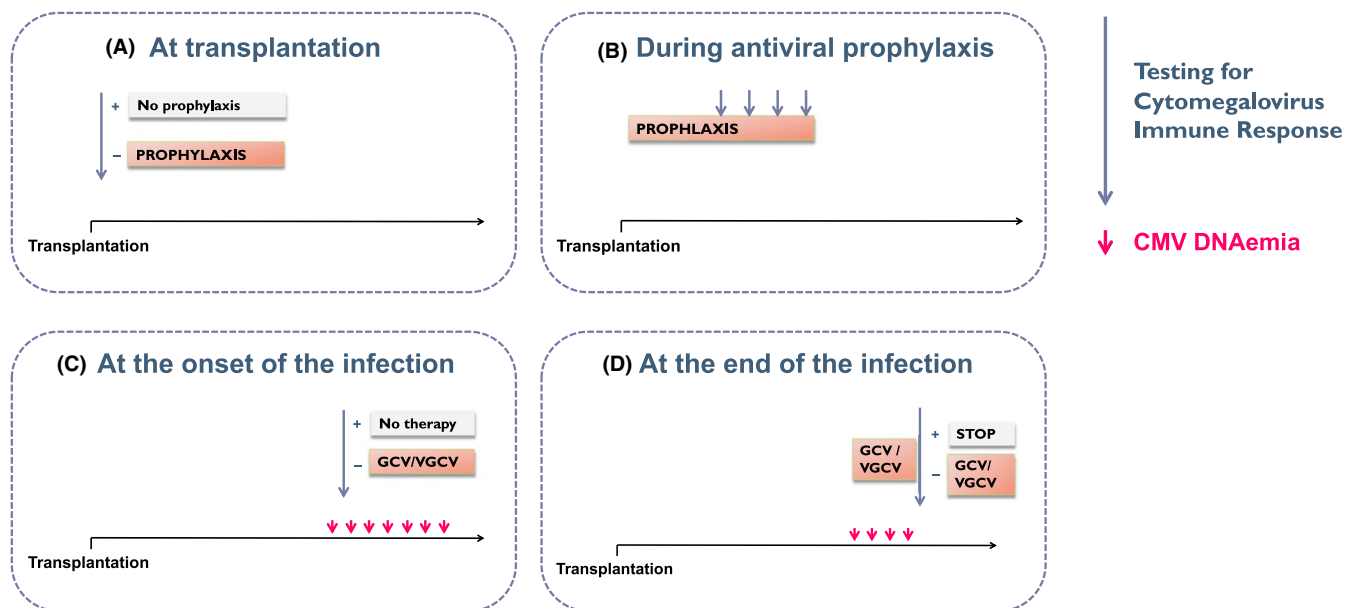
FIGURE 4 Clinical scenarios in which monitoring the cytomegalovirus-specific immune response would inform clinical decision. A, At the beginning of transplantation to predict the risk of CMV infection or disease. Preventive treatment could be avoided in patients with a protective CMV-specific immune response. B, During antiviral prophylaxis to determine for how long it is needed. Prophylaxis could be shortened in patients developing a protective CMV-specific immune response. C, At the beginning of CMV infection in asymptomatic patients. Patients can have a spontaneous viral clearance if they have a protective CMV-specific immune response. On the opposite, a curative treatment is mandatory in those without CMV-specific immune response. D, At the end of a curative antiviral treatment to predict recurrence and determine the necessity of a secondary prophylaxis in patients without CMV-specific immune response. GCV: ganciclovir, VGCV: valganciclovir

avoid useless CMV curative therapy in some cases of CMV infection in patients who have a protective CMV-specific αβ T CMI.

6.2.4 | At the end of a curative antiviral treatment for predicting recurrence

A prospective study performed at the end of CMV disease explored the utility of secondary prophylaxis after CMV DNAemia clearance using QuantiFERON-CMV assessment. No prophylaxis was given in case of positive QuantiFERON-CMV (>0.2 IU/ml), whereas an extended secondary prophylaxis was given in case of negative QuantiFERON-CMV to avoid CMV recurrence. The authors showed that 93% of patients with a positive QuantiFERON did not undergo CMV infection recurrence, in the absence of secondary prophylaxis. Conversely, 69.2% of patients with a negative QuantiFERON-CMV underwent CMV DNAemia recurrence, despite two months of secondary prophylaxis.[33] A protective CMV-specific αβ T CMI is then able to predict the absence of recurrence.

6.3 | CMV-specific αβ T cell monitoring in HCT recipients

6.3.1 | At the beginning of transplantation or after universal prophylaxis discontinuation

Recently, T-SPOT was evaluated in 241 CMV-seropositive allo-HCT recipients. Patients were monitored from the pretransplant period until six months post-transplantation. Clinically significant CMV infection (CS-CMVi), defined by CMV viremia or disease requiring antiviral treatment, was evaluated. 94%-96% of patients with CS-CMVi had a low CMV-ELISPOT. Conversely, among patients without CS-CMVi, 32% had a high CMV-ELISPOT.[223] Importantly, additional studies found an association between low levels of CMV-specific αβ T cells, evaluated both by QuantiFERON-CMV and by T-SPOT, and the risk of post-transplant reactivation.[224-226]

6.3.2 | In case of positive low-level CMV DNAemia

El-Haddad and colleagues followed 55 allo-HCT recipients who had experienced a first episode of low-level CMV DNAemia. They monitored CMV-specific αβ T cells by T-SPOT weekly and assessed the progression from a low-level CMV reactivation to the onset of CMV end-organ disease within 60 days of enrollment, and the subsequent initiation of CMV antiviral therapy by the treating physician in accordance. They assessed a cutoff for both IE1 and pp-65 ELISPOT. Progression occurred in 56% of patients, and the results of T-SPOT assays for the pp65 and IE1 antigens were significantly lower in patients who experienced this progression, compared with patients who did not. However, patients with the highest risk of CMV disease (patients undergoing haploidentical and cord blood transplantation) were excluded.[227]

Conversely, Gimenez and colleagues included high-risk recipients, and considered subsequent CMV episodes after the first one. They did not find a positive correlation between intracellular IFN-γ staining in CD8+ cells and CMV disease.[228] Thus, these studies did not use the same CMV-specific αβ T cell measurement nor the same inclusion criteria and found contradictory results. Moreover, they were both retrospective. Prospective studies with validated immune measurements are needed to be of clinical guide in this scenario.

6.3.3 | During CMV disease

So far, no study has evaluated the input of CMV-specific T cell monitoring during CMV disease to guide the duration of curative treatment.

6.4 | NK cell monitoring

NK cells contribute to early immune responses to viral infections,[229] and CMV seropositivity has been associated with the expansion in the peripheral blood of a population of NKG2C+ NK cells.[230-232] In CMV-seropositive patients, these expanded NKG2C+ CD56dim NK cells are highly differentiated, with high expression of granzyme B notably, show a polyfunctional response (expression of CD107a, IFN-γ, and TNF-α) to stimulation with HLA-E–expressing target cells, and harbor a clonal expression pattern of KIRs.[233] Altogether, this subset of clonotypic NK cells has been described as adaptive NK cells.[35,43,45,233]

In KTR[230] and in cord blood or HCT recipients,[231,232] CMV reactivation was shown to promote NKG2C+ NK cell expansion. More precisely, the presence of a CMV-seropositive hematopoietic cell allograft was correlated with a higher adaptive NKG2C+ NK cell expansion. This higher expansion was itself associated with better CMV disease-free survival.[234] Moreover, the number of NKG2C copies (1-2 versus 3-4) in double umbilical cord blood transplantation was inversely correlated with CMV reactivation post-transplantation (93.7 and 58.4%, respectively).[235] Conversely, Gimenez and colleagues observed that the absolute number of NKG2C(+) NK cells at day 30 post-HCT was not correlated neither with CMV DNAemia nor with the peak CMV DNA load within subsequent episodes of CMV DNAemia.[236] More recently, in a cohort of D+R− and R+ KTR without antiviral prophylaxis and without anti-thymocyte globulins, extended phenotyping on NK cells including NKG2C, NKG2A, CD57, ILT2, FcεRIγ, and PLZF was performed. High percentages of NKG2C+NKG2A− or NKG2C+ FcεRIγ− were associated with free CMV DNAemia post-transplantation, whereas a high percentage of NKG2C-NKG2A+ NK cells was associated with CMV DNAemia. Pretransplant percentages of NKG2C+NKG2A− NK cells were higher in patients with no viremia or with asymptomatic viremia compared with symptomatic patients. Finally, a cutoff was found for NKG2C+NKG2A− NK cells pretransplantation but the AUC (area under the curve) was 0.71 showing a non-optimal discrimination power.[237] Importantly, this extensive phenotyping of NKG2C+ NK helps understanding why the monitoring of NKG2C expression alone, without monitoring of co-inhibitory

receptor such as NKG2A, could fail to appraise the risk of CMV reactivation post-transplantation.

6.5 | Monitoring non-Vγ9Vδ2 T cells

As described earlier, non-Vγ9Vδ2 T cells undergo a specific expansion associated with a specific phenotypic signature during the course of CMV infection. Therefore, they could be complementary to the monitoring of CMV-specific αβ T CMI for managing CMV preventive or curative therapies.

6.5.1 | Non-Vγ9Vδ2 T cell monitoring in solid organ transplant recipients

At the time of kidney transplantation or during the first weeks post-transplantation, the percentage of peripheral blood Vδ2neg γδ T cells is not able to predict subsequent CMV infection or disease in R$^+$ patients (unpublished data). It is not surprising since Vδ2neg γδ T cell expansion has been previously observed after CMV infection in kidney transplant recipients. Moreover the occurrence of this expansion has been well correlated with CMV DNAemia clearance, both in D$^+$R$^-$ and in R$^+$ patients.[34,77,78,176,238] Consequently, we are currently evaluating the interest of monitoring this Vδ2neg γδ T cell expansion at the end of antiviral curative treatment to stop it if Vδ2neg γδ T cell expansion is present and to continue secondary prophylaxis up to three months until Vδ2neg γδ T cell expansion occurs, with a monthly monitoring (Clinicaltrial. gov, Kaminski NCT03339661).

6.5.2 | Non-Vγ9Vδ2 T cell monitoring in HCT recipients

In HCT recipients, non-Vγ9Vδ2 T cells have been poorly evaluated as an immunomonitoring tool. In a first study, Knight et al showed the Vδ2neg γδ T cell expansion in the peripheral blood of HCT recipient undergoing CMV infection compared with non-infected patients.[83] In a study including 105 recipients, it has been shown that the frequency of CD27$^+$ γδ T cells shortly after transplantation was inversely correlated with CMV reactivation.[178] Conversely, Barron and colleagues did not find any association between γδ T cell numbers and CMV reactivation, but measured CD69$^+$IFN-γ$^+$ γδ T cells and not total non-Vγ9Vδ2 T cells.[226]

6.5.3 | Perspectives for non-Vγ9Vδ2 T cell monitoring

In KTR, neither Vδ2neg γδ T cell percentage, nor TEMRA percentage among Vδ2neg γδ T cells at the beginning of transplantation (day 0, day 7, day 14) were found associated with the risk of CMV disease

(unpublished data). However, as we observed that Vδ2neg γδ T cells could express both inhibitory (eg, PD1, TIM3, LAG3, CD85j) and activating receptors (DNAM-1, CD8 αα, CD16), it would be highly informative to perform multiparametric flow cytometry analyses at the beginning of transplantation, to evaluate whether a phenotypic profile could be associated with an either increased or decreased risk of CMV disease. Besides, no study so far analyzed non-Vγ9Vδ2 T cells at the end of prophylaxis to determine whether a specific profile could be predictive of CMV disease after antiviral prophylaxis discontinuation.

Since Vγ9negVδ2pos T cell expansion has been associated with severe forms of CMV infection in KTR, it would be interesting to monitor this subset during the course of CMV infection, in order to identify patients who could benefit from immunosuppression modulation.

Moreover, transplantation of old patients is increasing and age is often a risk factor for CMV disease post-transplantation.[221,238] As old CMV-seropositive individuals have higher TEMRA Vδ2neg γδ T cells, it would be informative to assess whether a specific profile of senescent or dysfunctional non-Vγ9Vδ2 T cells could be associated with an increased risk of CMV reactivation after transplantation.

Altogether, a combined score measuring both non-Vγ9Vδ2 T cells and CMV-specific αβ T cells in the four clinical scenarios described above (Figure 4) could be of interest in order to improve both the sensitivity and specificity of cell-mediated immunity monitoring.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2

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Human γδ T cells comprise a first line of defense through T-cell receptor (TCR) recognition of stressed cells. However, the molecular determinants and stress pathways involved in this recognition are largely unknown. Here we show that exposure of tumor cells to various stress situations led to tumor cell recognition by a Vγ8Vδ3 TCR. Using a strategy that we previously developed to identify antigenic ligands of γδ TCRs, annexin A2 was identified as the direct ligand of Vγ8Vδ3 TCR, and was found to be expressed on tumor cells upon the stress situations tested in a reactive oxygen species-dependent manner. Moreover, purified annexin A2 was able to stimulate the proliferation of a Vδ2^{neg} γδ T-cell subset within peripheral blood mononuclear cells and other annexin A2-specific Vδ2^{neg} γδ T-cell clones could be derived from peripheral blood mononuclear cells. We thus propose membrane exposure of annexin A2 as an oxidative stress signal for some Vδ2^{neg} γδ T cells that could be involved in an adaptive stress surveillance.

gamma-delta T cells | innate-like lymphocytes | cell stress surveillance | tumor immunology | cytomegalovirus

It is well established that one of the major roles of conventional αβ-T lymphocytes is to protect the host against microorganisms. The molecular cornerstone of this function is the recognition by their antigen receptor of microbial moieties presented in the context of classic MHC molecules (1). In contrast, γδ T lymphocytes do not recognize peptides presented by classic MHC molecules and are biased against self-reactive recognition. Consistent with the Ig-like structure of γδ T-cell receptors (TCRs) (2) and the diversity of their repertoire, the self-antigens so far described to be directly recognized by γδ TCRs are structurally highly diverse, including MHC-related or unrelated molecules (for recent reviews, see refs. 3 and 4). Intriguingly, most of those self-antigens are constitutively expressed on cells and healthy tissues, implying mechanisms to control the γδ T-cell response in appropriate situations and avoid autoimmunity. Some of these mechanisms have been described, such as increased self-antigen expression upon cell activation (e.g., T10/T22 in mice) (5), dependence of recognition on a multimolecular stress signature in CMV-infected cells and tumor cells [endothelial protein C receptor (EPCR)] (6), presentation of—or conformational modification by—metabolites [CD1d (7, 8) and BTN3A1 (9–11)], and ectopic localization in tumor cells (F1-ATPase/ApoI) (12).

Although the pathophysiological contexts associated with the expression of these self-antigens in the appropriate environment or conformation leading to γδ T-cell response remains elusive for most of them, the contribution of γδ T cells to host protection is thought to rely on recognition of cell dysregulation. The so-called lymphoid stress surveillance response has been described as rapid, weakly specific, and resulting from activation of large numbers of preactivated or preprogrammed γδ T cells without necessary clonal expansion (13). Stress surveillance by γδ T cells is considered important for tissue repair (14), rapid local

containment of microbes or tumors (15–17), and activation of downstream conventional immune responses (18).

Given their implication in the control of tumors and infections, understanding the molecular basis of stress surveillance by γδ T cells could have important impacts on their use in immunotherapy. Such understanding has been hindered by the limited characterization of bona fide stress-induced antigens recognized by γδ TCRs and of the stress pathways leading to the expression of these antigens. The objective of the present study was to provide novel insights into these issues. We focused on Vδ2^{neg} γδ T-cell clones isolated from healthy donors previously shown to react against a broad panel of B-cell lymphoma in an Ig-like transcript (ILT)-2–dependent pathway (19). We elucidate herein the antigenic specificity of one of these clones as being annexin A2, a molecule expressed on the cell surface in response to oxidative stress and able to activate a subset of Vδ2^{neg} γδ T cells.

Results

Expression of 73R9 Ligand by U373MG Glioblastoma Cell Line. We focused on the Vγ8Vδ3 T-cell clone (73R9) that was reactive against transformed B cells (19). HLA-I engagement on 73R9 by

Significance

Human γδ T lymphocytes have innate-like and adaptive-like functions and can circulate in blood or reside in tissues. They are activated by specific antigens recognized by their T-cell receptor and recognize infected and transformed cells, suggesting that cellular stress is involved in specific antigen expression. However, molecular characterization of stress-induced antigens remains elusive, hampering our understanding of the role of γδ T cells in cancer and infections. In the present study we identify annexin A2 as such stress-induced antigen known as a phospholipid-binding protein involved in tumorigenesis, redox potential regulation, and wound healing. Stress-mediated membrane exposure of annexin A2 could thus constitute a danger signal for γδ T cells to recognize various cell dysregulations and protect the host against cancer and infections.
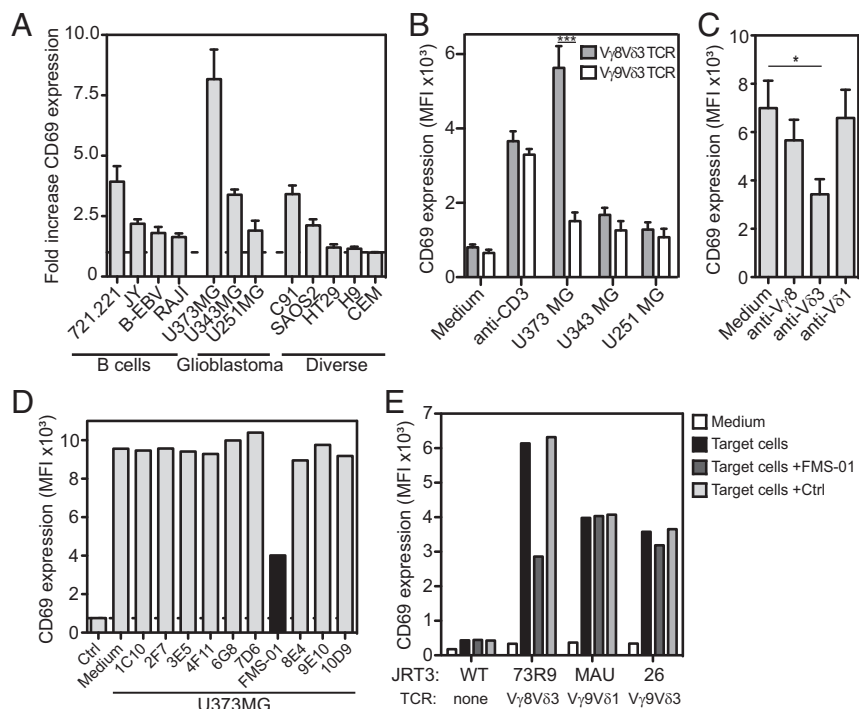
IMMUNOLOGY AND INFLAMMATION

Fig. 1. FMS-01 mAb inhibits 73R9 TCR-mediated recognition of U373MG. (*A*) CD69 expression by JRT3-73R9 cocultured 4 h with different target cells. Results shown are fold-increase of CD69 mean fluorescence intensity (MFI) in the presence of target cells versus in medium alone (horizontal dotted line). CEM, cell line donor's initials. (*B*) CD69 expression by JRT3-73R9 (Vγ8Vδ3 TCR) and JRT3-26 (Vγ9Vδ3 TCR) cocultured 4 h with glioblastoma cells with anti-CD3 mAb. (*C*) CD69 expression by JRT3-73R9 incubated with U373MG and with or without anti-Vγ8, anti-Vδ3, or anti-Vδ1 mAbs. In *A*–*C* bars represent the mean ± SEM of at least three independent experiments and Mann–Whitney test was used to compare conditions (*$P <$ 0.05, ***$P <$ 0.001). (*D*) CD69 expression by JRT3-73R9 incubated alone (Ctrl) or with U373MG cells in the presence or absence of a selection of hybridoma supernatants. (*E*) CD69 expression by JRT3 reporter cells expressing no TCR (JRT3 WT) or indicated Vδ2[neg] γδ TCRs, cultured in medium alone or with their own target cells (U373MG, HT29, SKW6.4). Supernatant of FMS-01 or control hybridoma (25% of culture volume) were added in indicated conditions. Data are representative of at least three independent experiments.

ILT-2 expressed on B cells was previously shown to stimulate clone cytolytic function (19). However, the role of the TCR in 73R9 recognition of B cells remained unclear. To address this issue we transduced the 73R9 TCR into the TCR-deficient human JRT3 T-cell, producing the JRT3-73R9 reporter cell line. Unexpectedly, when assayed against transformed B-cell lines the activation of JRT3-73R9 was markedly low (Fig. 1*A*), except against the 721.221 cell line. Among 42 other tumor cell lines tested, only the U373MG glioblastoma cell line induced a strong JRT3-73R9 cell activation, which was TCR-specific because it was not observed with other Vδ3 TCRs and inhibited by blocking anti-Vδ3 chain mAb (Fig. 1 *A*–*C*). Two other glioblastoma cell lines (U343MG and U251MG) also weakly induced JRT3-73R9 cell activation (Fig. 1*A*). Taken together, our results suggested that, by contrast to transformed B cells, the U373MG cell line expressed an antigen specifically recognized by the 73R9 TCR.

Generation of a mAb Specifically Blocking 73R9 TCR Reactivity. To characterize 73R9 TCR antigenic ligand, we generated a specific blocking monoclonal antibody using the strategy we previously described (6). Mice were immunized with U373MG and B-cell hybridoma supernatants screened for their ability to decrease JRT3-73R9 reactivity against U373MG. Such a hybridoma was selected and cloned to produce a mAb called FMS-01 (Fig. 1*D*). Inhibition by FMS-01 was not observed for other γδ TCRs (Fig. 1*E*). Among glioblastoma, U373MG was the cell line constitutively expressing the most important level of antigen labeled by FMS-01 mAb (Fig. S1*A*) in accordance with JRT3-73R9 reactivity (Fig. 1*A*). We concluded that FMS-01 competed with 73R9 TCR and most likely recognized the same antigen.

In agreement with the discrepancy between the results obtained with JRT3-73R9 versus clone 73R9 on B-cell recognition, B lymphoma cells were found to express very low levels of FMS-01 antigen, except for 721.221 cells, compared with glioblastoma and C91 T-lymphoma cells (Fig. S1*A*). Conversely, B-cell lines expressed a high level of ILT-2, whereas C91 and glioblastoma cells did not (Fig. S1*A*). Accordingly, anti–ILT-2 mAb, but not FMS-01 mAb, inhibited activation of 73R9 by B cells, and inversed results were obtained when using C91 or U373MG cells (Fig. S1*B*). A slight additive effect of combining both mAbs on B-cell recognition suggested a low-grade 73R9 TCR engagement by B cells (Fig. S1*B*), which was consistent

with up-regulated B-cell–mediated activation of JRT3-73R9 when increasing the B-cell:JRT3-73R9 ratio (Fig. S1*C*). Taken together, these results indicated that the same γδ T-cell clone can use TCR-dependent or -independent pathways to respond to different target cells.

CMV-Induced 73R9 TCR Activation Through Up-Regulation of FMS-01 Ligand Expression. Our previous studies demonstrated that some Vδ2[neg] γδ T cells exhibit dual TCR-dependent reactivity against tumor cell lines and CMV-infected cells (20). In accordance with this finding, CMV infection of U373MG and U343MG—but not that of U251MG—significantly up-regulated 73R9 activation (Fig. 2*A*). This effect of CMV was TCR-dependent because it was also observed when using JRT3-73R9 (Fig. 2*B*), because it was inhibited by FMS-01 mAb (Fig. S2*A*) and associated to increased FMS-01 ligand expression on U373MG (Fig. 2*C*). These
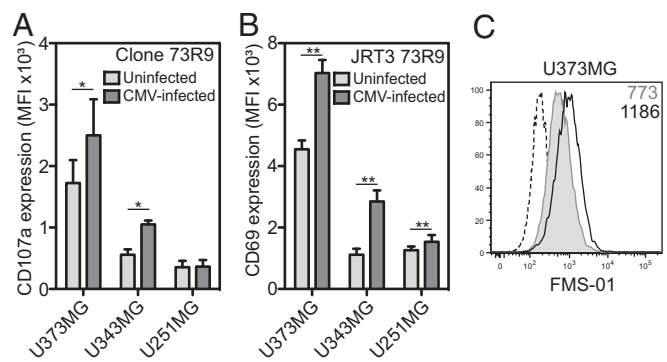
Fig. 2. CMV infection of glioblastoma cells enhances 73R9 TCR reactivity and FMS-01 expression. Activation of clone 73R9 (*A*) or JRT3-73R9 (*B*) by coculture with CMV-infected or uninfected glioblastoma cells. (*C*) Cell surface staining of CMV-infected (black line) and uninfected (gray histogram) U373MG with FMS-01, or with GAM IgM (dotted line). Results are mean ± SEM (*A* and *B*) or representative (*C*) of at least three experiments. Statistical significance was tested using the Willcoxon test, *$P <$ 0.05 and **$P <$ 0.005.
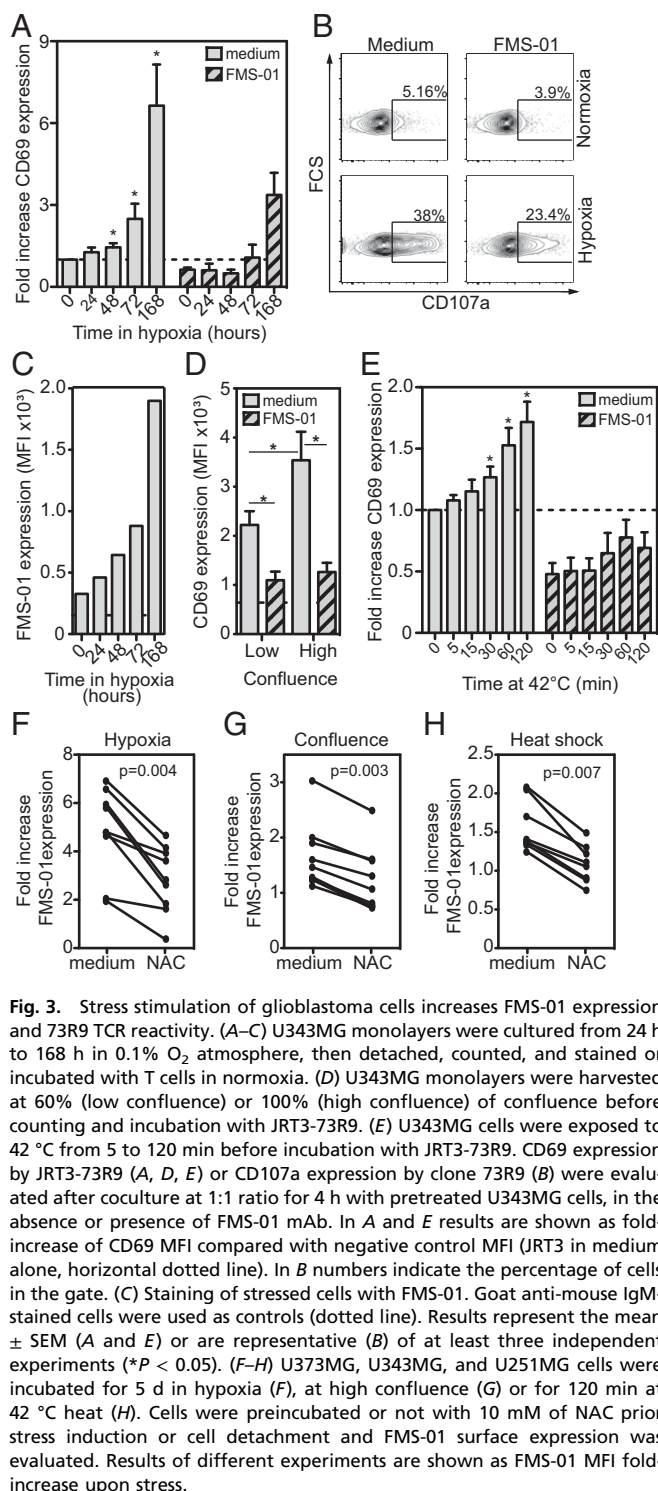
Fig. 3. Stress stimulation of glioblastoma cells increases FMS-01 expression and 73R9 TCR reactivity. (A–C) U343MG monolayers were cultured from 24 h to 168 h in 0.1% O_2 atmosphere, then detached, counted, and stained or incubated with T cells in normoxia. (D) U343MG monolayers were harvested at 60% (low confluence) or 100% (high confluence) of confluence before counting and incubation with JRT3-73R9. (E) U343MG cells were exposed to 42 °C from 5 to 120 min before incubation with JRT3-73R9. CD69 expression by JRT3-73R9 (A, D, E) or CD107a expression by clone 73R9 (B) were evaluated after coculture at 1:1 ratio for 4 h with pretreated U343MG cells, in the absence or presence of FMS-01 mAb. In A and E results are shown as fold-increase of CD69 MFI compared with negative control MFI (JRT3 in medium alone, horizontal dotted line). In B numbers indicate the percentage of cells in the gate. (C) Staining of stressed cells with FMS-01. Goat anti-mouse IgM-stained cells were used as controls (dotted line). Results represent the mean ± SEM (A and E) or are representative (B) of at least three independent experiments (*P < 0.05). (F–H) U373MG, U343MG, and U251MG cells were incubated for 5 d in hypoxia (F), at high confluence (G) or for 120 min at 42 °C heat (H). Cells were preincubated or not with 10 mM of NAC prior stress induction or cell detachment and FMS-01 surface expression was evaluated. Results of different experiments are shown as FMS-01 MFI fold-increase upon stress.

results supported the hypothesis that CMV-induced stress in host cells increased antigenic ligand expression and subsequent TCR-mediated activation of γδ T cells.

Different Cell Stress Conditions Trigger 73R9 TCR Reactivity. We then investigated other conditions of cellular stress that could modulate target cell recognition by γδ T cells. First, preincubation of U343MG in hypoxia (0.1% O_2) induced both JRT3-73R9 and 73R9 clone reactivity compared with preincubation in normoxia (Fig. 3 A and B) (all activation assays were done at 21% O_2,

37 °C, and with the same number of target cells). JRT3 transduced with other γδ TCRs did not respond to hypoxia-treated glioblastoma cells. Hypoxia-induced JRT3-73R9 and 73R9 clone activation was inhibited by FMS-01 mAb (Fig. 3 A and B) and associated to increased FMS-01 ligand expression on U373MG cells (Fig. 3C), suggesting γδ TCR-mediated stress sensing. Similar results were obtained when U343MG were pre-exposed to high confluence (Fig. 3D and Fig. S2 B and C) or to heat shock (Fig. 3E and Fig. S2 D and E), and when using U373MG or U251MG. TCR 73R9 activation always consistently correlated with FMS-01 staining on the target cell surface for each of these stress conditions (Table S1). Oxydative burst could be a common trigger to induce FMS-01 ligand expression because treating glioblastoma cells with the free radical scavenger N-acetyl-L-cysteine (NAC) during stress exposure partially inhibited the increased FMS-01 ligand expression by all stress conditions tested on the three glioblastoma cell lines (Fig. 3 F–H). In conclusion, different cell-stress conditions enhance γδ TCR-mediated sensing of target cells through an increased expression of membrane ligand, which is at least partially dependent on reactive oxygen species (ROS) production.

Identification of Annexin A2 as the Ligand for FMS-01 mAb. The nature of the membrane moiety bound by FMS-01 was then identified through immunoprecipitation. FMS-01 specifically immunoprecipitated a protein of ~35 kDa from all glioblastoma cell lysates but not from a FMS-01⁻ control cell line (Fig. 4A). Proteins contained within the specific ~35-kDa band were
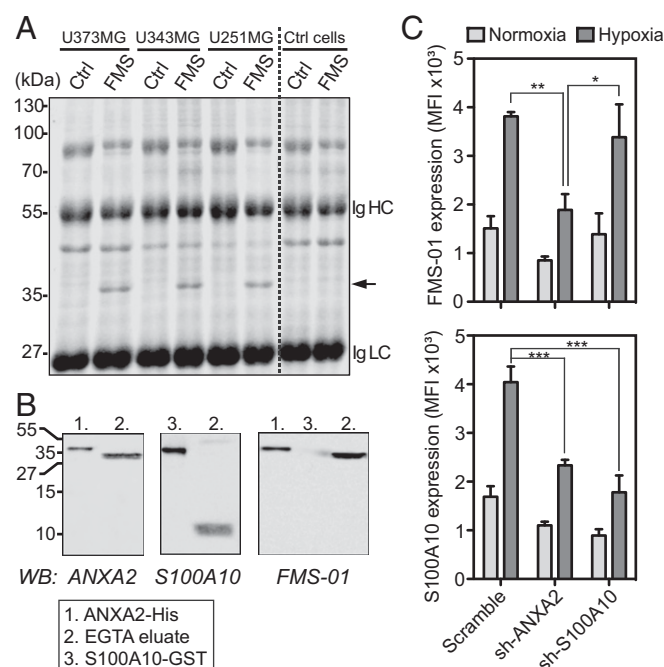
Fig. 4. FMS-01 recognizes annexin A2. (A) Colloidal blue-stained SDS/PAGE of proteins immunoprecipitated with control IgM (Ctrl) or FMS-01 mAb (FMS) from glioblastoma or FMS-01⁻ cells (Ctrl cells). Black arrow indicates the specific band. Heavy (Ig HC) and light chains (Ig LC) of antibodies used for immunoprecipitation are indicated. (B) Immunoblot analysis of EGTA eluates from U373MG, recombinant annexin 2 (His-tagged), and recombinant S100A10 (GST-tagged), detected with anti-annexin 2 mAb (Left), anti-S100A10 mAb (Center), or FMS-01 mAb (Right). Data are representative of at least two independent experiments. (C) Expression of FMS-01 ligand (Upper) and S100A10 (Lower) by U373MG transduced with scramble, annexin A2 (ANXA2), or S100A10 sh-RNAs and treated in normoxia or hypoxia. Data are represented as mean ± SEM of three independent experiments and two-way ANOVA test was used to compare conditions (*P < 0.05, **P < 0.005, ***P < 0.001).
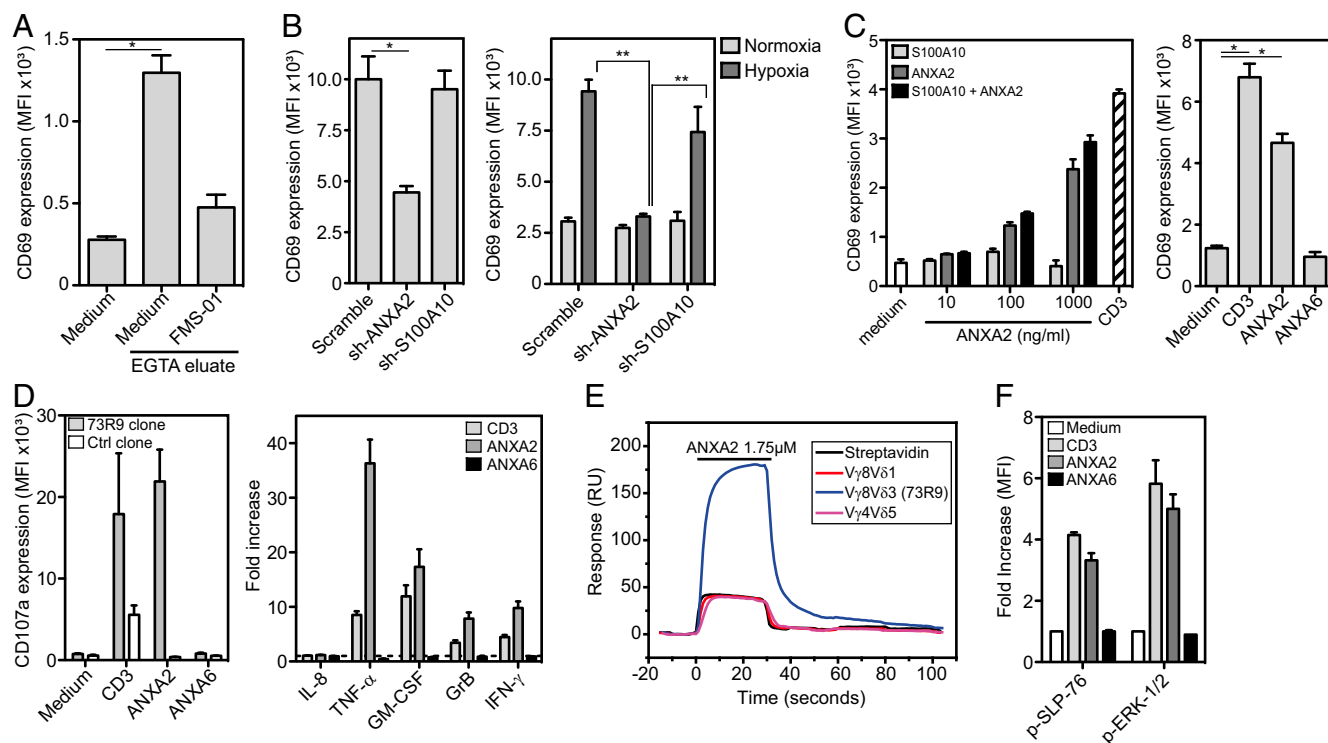
Fig. 5. 73R9 TCR recognizes annexin A2 CD69 expression by JRT3-73R9 incubated: (*A*) with or without EGTA eluates from highly confluent U373MG cells with or without FMS-01 mAb; (*B*) with U373 (*Left*) or U343 (*Right*) transduced with scramble, annexin A2 (ANXA2), or S100A10 sh-RNAs and pretreated in normoxia or hypoxia; (*C*) with or without increasing doses of recombinant soluble annexin A2 and/or S100A10 (*Left*), with anti-CD3 mAb, or with soluble annexin A6 (*Right*). (*D*) Clone 73R9 was activated with anti-CD3 mAb, soluble annexin A2 or A6, and CD107a membrane expression after 4 h (*Left*) or indicated cytokine secretion after 24 h (*Right*) were evaluated by flow cytometry. (*E*) Binding of annexin A2 (1.75 μM) to biotinylated 73R9 TCR or two control TCRs immobilized on streptavidin-coated flow cells at 2,153 RU, 2,175 RU, and 2,748 RU, respectively, or streptavidin alone, assessed by SPR and presented as resonance units (RU). (*F*) Detection of phosphorylated SLP-76 and ERK-1/2 in clone 73R9 incubated in the indicated conditions. All of the results are from at least three independent experiments and are shown as mean ± SEM (*$P < 0.05$, **$P < 0.005$).

digested with trypsin and analyzed by Fourier transform-ion-cyclotron resonance mass spectrometry. This process identified annexin A2, a 35-kDa intracellular protein known to bind anionic phospholipids in a Ca^{2+}-dependent manner, and to translocate to the cell surface as a heterotetrameric complex with the 11-kDa protein S100A10 (21). In line with this finding, FMS-01 staining strongly correlated with S100A10 expression in different cell types (Fig. S3*A*). Moreover, proteins immunoprecipitated with FMS-01 mAb were also detected with anti-annexin A2 and anti-S100A10 mAbs by Western blots (Fig. S3*B*). Western blots using recombinant forms of both proteins, or U373MG EGTA eluates containing annexin A2/S100A10 complex from the cell surface, as previously shown (21), demonstrated that FMS-01 bound annexin A2 only (Fig. 4*B*). Finally, down-regulation of annexin A2 or S100A10 expression in glioblastoma cell lines using specific sh-RNA showed that surface staining by FMS-01 was dependent on annexin A2 expression but independent of S100A10 expression (Fig. 4*C* and Fig. S3*C*).

Annexin A2 Is Recognized by the 73R9 TCR. FMS-01 ligand was expected to be 73R9 TCR ligand, it was then important to ensure that 73R9 TCR recognized annexin A2. EGTA membrane eluates from U373MG cells cultured at high confluence were able to activate JRT3-73R9, but not other TCR-transductants, in an annexin A2-dependent manner, in contrast to eluates generated from cells cultured at low confluence (Fig. 5*A* and Fig. S4*A*). Annexin A2 was mandatory for recognition of glioblastoma cells by JRT3-73R9 because down-regulation of its expression by RNA interference abrogated JRT3-73R9 activation, whereas down-regulation of S100A10 had no effect (Fig. 5*B*). Remarkably, recombinant soluble annexin A2 alone, but not recombinant

S100A10, was able to activate very efficiently JRT3-73R9 (Fig. 5*C*, *Left*). Activation was not observed when using annexin A6 (Fig. 5*C*, *Right*) or another TCR-transductant (Fig. S4*B*), and was inhibited in the presence of the FMS-01 mAb (Fig. S4*C*). Moreover, soluble annexin A2 was at least as efficient as anti-CD3 mAb to activate the 73R9 clone (Fig. 5*D*). Finally, annexin A2 but not A6 was able to induce multiple functions on the clone such as cytotoxicity (assessed by CD107a expression and granzyme B production) but also TNF-α, IFN-γ, and GM-CSF secretion (Fig. 5*D*).

Molecular interaction between annexin A2 and the 73R9 TCR was then confirmed by surface plasmon resonance (SPR). We observed greater responses when recombinant annexin A2 was injected over immobilized 73R9 TCR compared with control TCRs or streptavidin alone, indicating specific binding (Fig. 5*E*). Equilibrium binding analyses yielded an apparent dissociation constant (K_d) of ~3 μM (Fig. S4*D*). No specific binding of annexin A6 was observed to 73R9 TCR compared with control TCR. Annexin A2 was not only binding but also signaling through the TCR because ERK 1/2 and SLP76 phosphorylation was induced in the 73R9 clone (Fig. 5*F*)—but not control clone (Fig. S4*E*)—as well as in the JRT3-73R9, but not in a control transductant (Fig. S4*F*). Taken together, these results provide evidence that the 73R9 TCR directly recognizes annexin A2 independently of S100A10. Annexin A2 translocation to the cell surface represents a unified stress signal recognized by this TCR.

Annexin A2 Induces the Proliferation of a Vδ2^neg γδ T-cell Subset. Finally, we tested the effect of annexin A2 on Vδ2^neg γδ T cells isolated from the blood of healthy donors. When cocultured with autologous peripheral blood mononuclear cells (PBMC), a

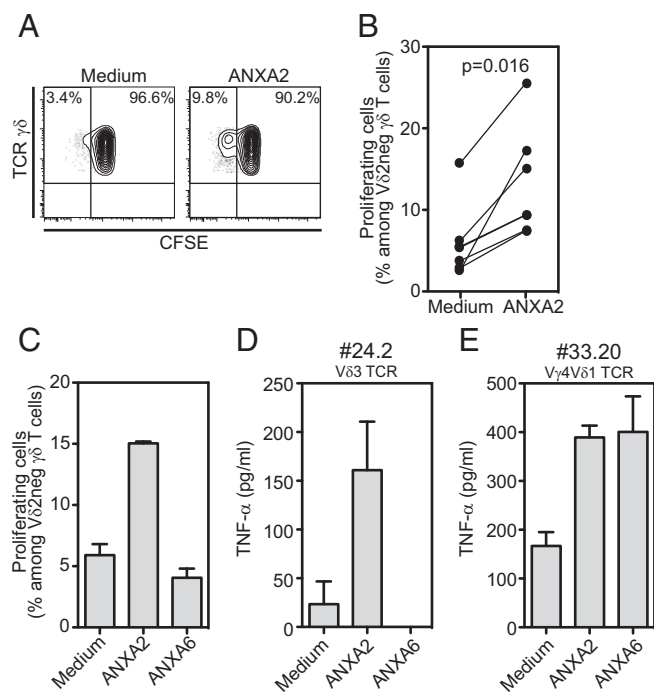**Fig. 6.** Annexin A2 stimulates $V\delta2^{neg}$ $\gamma\delta$ T-cell proliferation. (*A–C*) CFSE [5-(and 6)-carboxyfluorescein diacetate succinimidyl ester] labeled $V\delta2^{neg}$ $\gamma\delta$ T cells were cocultured with autologous PBMC in the presence of recombinant IL-2 with or without annexin A2 for 5 d. Results are presented as percentages of CFSE-low cells among $V\delta2^{neg}$ $\gamma\delta$ T cells. (*A*) Representative dot-plot of flow cytometry staining, (*B*) percentages of proliferating $V\delta2^{neg}$ $\gamma\delta$ T cells from independent donors (n = 7), and (*C*) comparison of annexin A2 and annexin A6 effects. (*D*) TNF-α production by two T-cell clones isolated from healthy donors and incubated for 24 h with soluble annexins A2 or A6. Results from *C–E* are mean ± SEM of at least two independent experiments.

small population of proliferating $V\delta2^{neg}$ $\gamma\delta$ T cells appeared in the presence of annexin A2 plus IL-2 compared with IL-2 alone (Fig. 6*A*). Results obtained from seven different donors are shown in Fig. 6*B*, indicating statistically significant increase of proliferating $V\delta2^{neg}$ $\gamma\delta$ T cells with annexin A2. Annexin A6 had no effect (Fig. 6*C*), in agreement with the results obtained with JRT3 73R9 activation and SPR. This finding prompted us to try to derive new annexin A2-specific $V\delta2^{neg}$ $\gamma\delta$ T-cell clones. From 72 $V\delta2^{neg}$ $\gamma\delta$ T-cell clones expanded polyclonally from three different healthy donors, two clones were able to react to annexin A2. Interestingly, one of them also reacted to annexin A6, suggesting the recognition by this clone of a region shared by both annexins. One clone (no. 24.2) expressed a $V\delta3$ TCR (and neither $V\gamma4$, $V\gamma8$, nor $V\gamma9$), and the other one (no. 33.20) expressed a $V\gamma4V\delta1$ TCR. These results indicate that annexin A2 specificity is not restricted to the 73R9 TCR or to $V\gamma8V\delta3$ TCRs.

Discussion

Because they are able to react to infected, activated, or transformed cells, and are involved in host response to diverse situations of stress, $\gamma\delta$ T cells are considered to be important players in lymphoid stress surveillance. However, the nature of the cellular dysregulation events that they respond to and the specific molecular stress stimuli that trigger their activation remain poorly understood. In particular, identification of the molecular signals associated with these dysregulations and specifically recognized by the $\gamma\delta$ TCR is still limited. As a contribution to this knowledge, we characterized annexin A2 translocation to the cell surface as a common molecular stress signal recognized by a $V\gamma8V\delta3$ TCR.

The $V\gamma8V\delta3$ $\gamma\delta$ T-cell clone 73R9 used in this study is representative of a panel of $V\delta2^{neg}$ $\gamma\delta$ T cells previously described to recognize a large panel of B-lymphoma cell lines through an atypical ILT-2/HLA axis (19). We show here that stressed glioblastoma cells can also activate clone 73R9. Interestingly, different molecular mechanisms mediated recognition of distinct target cells. $\gamma\delta$ T-cell HLA molecules recognize ILT-2 on B-lymphoma cells and the $V\gamma8V\delta3$ TCR is not (or weakly) involved in this process. In contrast, the TCR recognizes annexin A2 on glioblastoma cells and ILT-2 is not involved. The same $\gamma\delta$ T cells can thus recognize different types of cellular dysregulation through distinct molecular pathways, making them able to integrate several and potentially separate contextual signals in order for them to enlarge their functional diversity and responses to different situations.

Here, we identify annexin A2 as the antigen targeted by FMS-01 mAb that specifically inhibited $V\gamma8V\delta3$ TCR-mediated recognition of glioblastoma cells. Together with the observation that purified annexin A2 was able to activate the $V\gamma8V\delta3$ TCR specifically, this result demonstrates that annexin A2 is critical for $V\gamma8V\delta3$ TCR-dependent recognition of target cells. Annexin A2 belongs to the evolutionary ancient family of Ca^{2+}-regulated phospholipid-binding annexin proteins (22). Annexin A2 is present in the cytoplasm, and is associated with intracellular membranes of different organelles and with the internal or extracellular face of plasma membrane. It participates in a variety of membrane-related functions (endocytosis, exocytosis, membrane repair) in response to diverse cellular fluctuations, including Ca^{2+} influx, pH variation, membrane phospholipid composition, and its own posttranslational modification. It can exist as a monomer or as heterotetrameric complexes with the S100A10 protein, which enhances its membrane phospholipid binding affinity. In our hands, the highest expression of annexin A2 observed at the cell surface was achieved by placing cells under hypoxia, probably because it combines both membrane translocation and an increase in annexin A2 gene expression, which has been shown to be dependent on HIF-1 (23). Cellular reoxygenation after hypoxia is followed by ROS burst, and inhibiting ROS production using antioxidant NAC decreased stress-induced annexin A2 surface expression. Oxidative stress could thus be a common pathway leading to annexin A2 membrane translocation and $\gamma\delta$ T-cell activation because NAC also decreased heat shock and high confluence-induced annexin A2 expression.

Several features of annexin A2 fulfill what we can expect from a canonical ligand of a $V\delta2^{neg}$ $\gamma\delta$ TCR. First, annexin A2 is overexpressed in many cancer cells, including glioblastoma, where it correlates positively with histologic grade and central nervous system dissemination (24). Second, despite the absence of a transmembrane domain, intracellular annexin A2 can swiftly translocate to cell surface upon stress signals (25) in agreement with the increase of FMS-01 binding on glioblastoma cells treated for only 30 min at 42 °C. In endothelial cells, annexin A2 translocation is obtained, in vitro but also in vivo, within minutes in response to heat stress, thrombin exposure, or hypoxia and relies on annexin A2 phosphorylation (21–23). Third, consistent with $\gamma\delta$ T-cell responses to tissue injury (26) annexin A2 plays a role in membrane repair and wound healing (27), which is supposedly because of an intracellular rise in Ca^{2+} upon membrane damage (28). Fourth, in agreement with 73R9 TCR recognition of CMV-infected cells, CMV-infection has been shown to induce annexin A2 phosphorylation, which is necessary for translocation to cell surface, for binding to CMV and to further enhance CMV infection (29).

Annexin A2 appears to represent a bona fide stress antigen expressed on the cell surface only upon cellular dysregulation, and able to alert $\gamma\delta$ T cells, such as 73R9, 24.2, or 33.20 $\gamma\delta$ T cells. Annexin A2-specific $\gamma\delta$ T cells could thus contribute to lymphoid stress surveillance, a property that has rather been so far attributed to innate-like invariant $\gamma\delta$ T cells (13). The diversity and low frequency of annexin A2-specific $\gamma\delta$ T cells that we describe in this study suggest that response to annexin A2 may rather represent an adaptive response requiring clonal expansion in specific situations. This "adaptive stress surveillance"

would probably be less immediately efficient than the massive response of invariant subsets but could be more rapid than a conventional αβ–T-cell response because of conceivably taking place within stressed tissues.

Annexins and S100 molecules have been previously classified among alarmins (30) because of their ability to induce inflammatory patterns in endothelial cells and macrophages. Annexin A2 could be considered as a γδ T-cell alarmin, acting either through cell–cell contact or as soluble form because annexin A2 can be released in the extracellular microenvironment (31). It is tempting to imagine that soluble annexin A2 could alert distant specific γδ T cells and stimulate their proliferation. However, the affinity of annexins for membrane phospholipids suggests that even when produced in soluble form, annexin A2 probably rapidly binds to proximal cell membranes and act in a membrane-bound fashion. Our results showing an induction of Vδ2^{neg} γδ T-cell proliferation by soluble annexin A2 should foster further investigations to evaluate the interest of this antigen in immunotherapeutic settings aiming at stimulating γδ T-cell control of cancer or infections.

Materials and Methods

For further details, see *SI Materials and Methods*.

Generation of Effector Cells. Human γδ T clone 73R9 (expressing a TCR Vγ8Vδ3) was obtained as previously described in ref. 19. Reporter cells expressing TCR 73R9 (JRT3-73R9) were generated as previously described (6) by cotransduction with viral particles expressing a Vγ8 TCR chain and particles expressing Vδ3 TCR chain. Amino acid sequences of the Vδ3-Dδ3-Jδ1 and Vγ8-Jγ2 junctional regions of 73R9 TCR are CAFTGLGDTSHADKLIF and CATWDSSKLFGSGTTLVVT, respectively.

Functional Assays with Stressed Cells. Activation of JRT3 transduced with γδ TCRs by tumor cell lines at 1:1 [effector cell:tumor cell (E:T)] ratio was measured by expression of CD69 by flow cytometry. Activation of clone 73R9 was analyzed using CD107a mobilization assay or cytokine production. TCR signaling was also analyzed by flow cytometry. In some experiments, tumor cells were infected with CMV clinical strain TB40/E for 4 d. Correct infection of the cells was confirmed by cytopathic effect observation. For hypoxic stress, tumor cells were grown in 21% or 0.1% oxygen atmosphere and were released, counted, and stained in normoxia. For heat-shock assay, cell lines were grown for 48 h, then detached and incubated at 42 °C or 37 °C for the indicated times. For all assays, target cells were washed twice before incubation with effector cells in a 1:1 ratio, or before staining with specific mAbs. In some experiments, glioblastoma cell lines were preincubated for 1 h with 10 mM NAC pH-adjusted solution, before stress induction (heat-shock) or cell detachment.

Generation of FMS-01 mAb. BALB/c mice were immunized with U373MG and hybridomas generated as previously described (6). Hybridomas that secreted antibody able to inhibit JRT3-73R9 reactivity against U373MG were cloned by limiting dilution, ending with selection of FMS-01 mAb because of its robust neutralizing activity. The experimental protocol was approved by Animal Care and Use Committee Board of Bordeaux (No. 50120124-A).

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Characterization of a Unique γδ T-Cell Subset as a Specific Marker of Cytomegalovirus Infection Severity

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Cytomegalovirus (CMV) is a major infectious cause of death and disease after transplantation. We have previously demonstrated that the tissue-associated adaptive Vδ2^{neg} γδ T cells are key effectors responding to CMV and associated with recovery, contrasting with their innatelike circulating counterparts, the Vγ9^{pos}Vδ2^{pos} T cells that respond to phosphoantigens but not to CMV. A third Vγ9^{neg}Vδ2^{pos} subgroup with adaptive functions has been described in adults. In the current study, we demonstrate that these Vγ9^{neg}Vδ2^{pos} T cells are also components of the CMV immune response while presenting with distinct characteristics from Vδ2^{neg} γδ T cells. In a cohort of kidney transplant recipients, CMV seropositivity was the unique clinical parameter associated with Vγ9^{neg}Vδ2^{pos} T-cell expansion and differentiation. Extensive phenotyping demonstrated their substantial cytotoxic potential and activation during acute CMV primary infection or reinfection. In vitro, Vγ9^{neg}Vδ2^{pos} T cells responded specifically to CMV-infected cells in a T-cell receptor–dependent manner and through strong interferon γ production. Finally, Vγ9^{neg}Vδ2^{pos} T cells were the only γδ T-cell subset in which expansion was tightly correlated with the severity of CMV disease. To conclude, our results identify a new player in the immune response against CMV and open interesting clinical perspectives for using Vγ9^{neg}Vδ2^{pos} T cells as an immune marker for CMV disease severity in immunocompromised patients.

Keywords. gamma-delta T cells; kidney transplant recipients; CMV infection.

Human cytomegalovirus (CMV) is a widespread herpesvirus. Although human CMV infection is asymptomatic in immunocompetent hosts, it induces organ damage during mother-to-child infection,and in immunocompromised patients, such as recipients of solid organ or hematopoietic stem cell allografts. In transplantation, the CMV serostatus of the donor and the recipient are key predictors of the risk and severity of infection and guide decisions on antiviral prevention. Despite those improvements in prevention strategies, CMV infection remains associated with increased posttransplantation morbidity and mortality rates [1]. A major challenge is to keep improving our knowledge of the actors involved in the anti-CMV immune response.

Besides the well-described role of αβ T cells to control CMV, we and others have demonstrated the contribution of γδ T cells. The human adult γδ T-cell repertoire has been classically divided into the Vγ9^{pos}Vδ2^{pos} and the Vδ2^{neg} T-cell compartments.

The Vγ9^{pos}Vδ2^{pos} T cells are innatelike cells predominant in the peripheral blood. They are activated by small phosphorylated metabolites of the isoprenoid synthesis pathways called phosphoantigens, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) [2]. The other γδ T cells are often pooled and collectively called Vδ2^{neg} γδ T cells because (1) they are all preferentially located in epithelial tissues, (2) they can express any Vδ chain, except Vδ2, in combination with any Vγ chain, and (3) they have been shown to respond to CMV (for review see [3]). After CMV infection [4], Vδ2^{neg} γδ T cells expand in the blood, kill CMV-infected cells in vitro [5, 6], and predominantly express a late differentiated effector phenotype called TEMRA (T effector memory CD45RA$^+$) [6–8], which led us to classify these cells in the adaptive compartment [7].

Even though Vδ2 chain has long been considered to associate only with Vγ9 chain, the existence of Vγ9^{neg}Vδ2^{pos} T cells has been reported, particularly in fetal thymus [9] and CMV-infected neonates [6]. They have also probably been underestimated because of technical flow cytometry issues regarding the type of anti-Vδ2 monoclonal antibodies and bad combinations of commercial monoclonal antibodies against pan-γδ T-cell receptors (TCRs), Vγ9, and Vδ2 hampering correct γδ TCR staining [10].

They also persist into adulthood peripheral blood, typically at low levels [11]. They express either Vγ2/3/4/5/8 chain, harbor an adaptive T-cell profile with a highly diverse TCR repertoire,

and have a more differentiated phenotype than their semi-invariant $V\gamma9^{pos}V\delta2^{pos}$ counterparts [12]. Moreover, from a functional perspective, this $V\gamma9^{neg}V\delta2^{pos}$ subset was only reported to be unresponsive to phosphoantigens [12].

Altogether, a parallel between $V\gamma9^{neg}V\delta2^{pos}$ and $V\delta2^{neg}$ T-cell subsets can be proposed, and it was suggested that they could both respond to CMV. To date, demonstrations of $V\gamma9^{neg}V\delta2^{pos}$ T-cell involvement in CMV immune response have been limited and rather indirect. $V\gamma9^{neg}V\delta2^{pos}$ T-cell switch from naive to effector memory phenotype and TCR clonal restriction were observed a long time after CMV seroconversion in only 2 organ recipients [12], and 3 $V\gamma9^{neg}V\delta2^{pos}$ clones have been shown to expand after CMV reinfection in 2 stem cell transplant recipients [13]. Moreover, the direct reactivity of those cells to CMV has never been assessed in either adults or neonates. The objectives of the present study were to ascertain the link between $V\gamma9^{neg}V\delta2^{pos}$ T cells and CMV infection, to demonstrate their specific reactivity against CMV, to define their distinct function from $V\delta2^{neg}$ γδ T cells. and consequently to clarify their clinical relevance within the anti-CMV specific immune response.

METHODS

Samples and Patients

Whole blood was obtained from 83 consecutive kidney transplant recipients admitted to Bordeaux University Hospital, for a systematic visit that includes a routine care γδ T-cell immunophenotyping. CMV serostatus for each kidney transplant recipients had been determined on the day of the graft and was retested for all CMV-seronegative (CMV−) kidney transplant recipients after transplantation. Blood was also obtained from 4 CMV− kidney transplant recipients in whom primary CMV infection developed after receipt of a transplant from a CMV-seropositive (CMV+) donor and 4 CMV+ kidney transplant recipients in whom reinfection developed (this term was used to define both superinfection or reactivation). For CMV and immunosuppressive regimen management, see the Supplementary Methods.

The research protocol was approved by the relevant local institutional review board (CPP Ouest IV; 50/17_1), and all kidney transplant recipients gave their written informed consent. We also retrospectively collected their clinical characteristics, using our local medical software (R@N), for which all kidney transplant recipients signed a written informed consent.

Flow Cytometry Analysis of γδ T Cells in Blood and Peripheral Blood Mononuclear Cells

Whole-blood staining was managed as described elsewhere [14, 15], with the following monoclonal antibodies: anti–pan-δ–phycoerythrin (PE), anti–Vδ2-PC7 (Miltenyi), anti–γ9 PC5 (Beckman Coulter), anti–CD3-V450, anti–CD27–allophycocyanin (APC) and anti–CD45RA–fluorescein isothiocyanate (FITC; BD Biosciences) (for complete reference on

antibodies, see the Supplementary Methods). BD FACS lysis solution (BD Biosciences; reference 349202) was used, and the samples were processed on a BD FACS Canto II cytometer (BD Biosciences). Peripheral blood mononuclear cells (PBMCs) were isolated by means of Ficoll density gradient. For each panel, 1 million PBMCs were incubated with the viability marker FSV575 (BD Biosciences), with monoclonal antibodies for CD3–peridinin-chlorophyll protein, Vδ2-PC-7 (Miltenyi). and either CD8-BV510 or PE–Texas red for the panel of cytotoxicity (BD Biosciences), and pan-δ–PE or pan-δ–APC (Miltenyi) for the cytotoxicity panel.

Then the staining included either CD85j-FITC, CD158a/b-BV421, CD161-BV650, CD16-BV786 (BD Biosciences), NKG2C-APC and KLRG1-PE-vio-615 (Miltenyi), or granulysin-FITC, FasL-BV650, TRAIL-BV510, Ki-67-BV786 and perforin-BV421 (BD Biosciences), granzyme PE (Molecular Probes); or programmed cell death 1 (PD-1)–BV650, TIM-3-BV711, CTLA4-APC, and DNAX accessory molecule (DNAM)-BV786 (all BD Biosciences). For complete reference on the antibodies and details on the PBMC staining procedure, see the Supplementary Methods. All PBMCs were processed using a BD LSRFortessa cytometer (BD Biosciences).

Expansion of γδ T Cells from Kidney Transplant Recipients' PBMCs

PBMCs were isolated from kidney transplant recipients undergoing CMV disease. γδ T cells were then expanded by culturing PBMCs in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% human serum, 1000 IU/mL recombinant human interleukin 2 (rIL-2; no. 200–02) and 10 ng/mL recombinant human interleukin 15 (rIL-15; no. 200–15) (all from Peprotech) for 7, 14, or 21 days, depending on the assay they were used for.

Preparation of CMV-, Herpes Simplex Virus–, or Varicella-Zoster Virus–Infected Fibroblasts and Free CMV/Herpes Simplex Virus/Varicella-Zoster Virus

CMV-, herpes simplex virus (HSV)–, and varicella-zoster virus (VZV)–infected fibroblasts and free viruses were prepared as described elsewhere [5] and are detailed again in the Supplementary Methods.

Proliferation Assays

After 14 days of culture, as described above, PBMCs were labeled with 5 μmol/L 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; no. V12883, eBioscience). Next, 4.10^5 CFSE-labeled PBMCs were cultured in 24-well plates, with, in each well, 800 μL of the same medium as above, or in the presence of noninfected or CMV-infected fibroblasts, with 100 IU/mL rIL-2 and 5 nmol/L HMBPP (HDMAPP; item 13580, no. 443892-56-6; Cayman Chemical), or with coated anti-CD3 as a positive control (OKT3; Ultra-LEAF purified [BLE317326; Ozyme]). For wells with rIL-15, 10 ng/mL rIL-15 was added after 4 days. After 7 days, cells were collected and incubated first with viability staining (fixable viability

stain 780; BD Biosciences), washed, and incubated with monoclonal antibodies against CD3-V450 (BD Biosciences), pan-δ–PE, Vδ2-PC7 (Miltenyi), and Vγ9-PC5 (Beckman Coulter). Then all cells from each sample were washed and processed with a BD LSRFortessa cytometer (BD Biosciences).

Interferon γ Assays

Intracytoplasmic PBMC Assays

After 7 days of culture, PBMCs were labeled with 1 μmol/L CFSE, and 4.10^5 cells were incubated in 24-well plates for 15 hours with complete RPMI 1640 medium (containing 8% fetal calf serum and 2 mmol/L glutamine; 800 μL per well), either alone or with noninfected fibroblasts, with CMV-infected fibroblasts, 100 IU/mL rIL-2 and 5 nmol/L HMBPP, or with precoated anti-CD3 (OKT3) as a positive control. PBMCs were stained with viability dye (fixable viability stain 575; BD Biosciences) and then with CD3-V450 (UCHT1; BD Biosciences), pan-δ–PE, Vδ2-PC7 (Miltenyi), and Vγ9 APC–cyanine 7 (Miltenyi). After fixation and permeabilization (as previously described [16]), cells were stained with BV786 anti-interferon (IFN) γ. For TCR-blocking experiments, anti-CD3-V450 was incubated with PBMCs for 15 minutes in phosphate-buffered saline at 4°C, and washed; PBMCs were then incubated as described above for 15 hours of culture and stained with the same panel. All PBMCs from each sample were processed with a BD LSRFortessa cytometer (BD Biosciences). The gating strategy is presented in Supplementary Figure 1.

Secretion in Supernatants From Purified γδ T Cells

After 21 days of culture (or directly from PBMCs for 1 patient), αβ T cells were eliminated from PBMCs through magnetic sorting (αβ–biotin and anti-biotin; Miltenyi Biotec), and then stained with anti-Vδ2-PC7 (Miltenyi Biotec) and anti-Vγ9-PC5 (Beckman Coulter). Vδ2^{neg}Vγ9^{pos} and Vδ2^{pos}Vγ9^{neg} cells were sorted (BD FACS Aria IIu cell sorter), with 98%–100% purity. After that, 10^4 cells were incubated per 96-well plate with RPMI 1640 medium with 8% fetal calf serum, 2 mmol/L glutamine, and 50 ng/mL rIL-18 [17] (MBL International; B003-5), alone or with either noninfected or CMV-infected fibroblasts for 24 hours at 37°C. Then the supernatants were collected for IFN-γ enzyme-linked immunosorbent assay operated, as described in the kit (human IFN-γ enzyme-linked immunosorbent assay development kit; Mabtech; no. 3420-1H-6).

Statistical Analysis

Mann-Whitney U tests, χ^2, or Fisher tests and unpaired t tests were used when appropriate. Differences were considered statistically significant at $P < .05$. Alternatively, paired t tests were used for paired data. Finally, Spearman correlation analysis was performed, using conventional statistical methods and GraphPad software, version 6.0, San Diego, CA. All figures were obtained with FlowJo software within t-distributed stochastic neighbor embedding (t-SNE) plugins [18] (v.10) and GraphPad Prism software.

RESULTS

Frequency and differentiation status of Vγ9^{neg}Vδ2^{pos} and Vδ2^{neg} subsets of γδ T cells are equally impacted by CMV serostatus

The frequency and differentiation status of Vγ9^{neg}Vδ2^{pos} and Vδ2^{neg} subsets of γδ T cells are equally affected by CMV serostatus In our cohort of 83 kidney transplant recipients, CMV+ (n = 59) and CMV− (n = 24) kidney transplant recipients did not differ except that anti-CMV universal prophylaxis was given more frequently in CMV− kidney transplant recipients (Table 1).

Both Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T-cell absolute counts were significantly higher in CMV+ kidney transplant recipients, whereas no difference was observed for Vγ9^{pos}Vδ2^{pos} T cells (Figure 1A and 1B). The percentages of Vδ2^{neg} T cells among total T cells (Figure 1B) or among γδ T cells (Supplementary Figure 2) were significantly higher in CMV+ than in CMV− patients, whereas a difference was observed for Vγ9^{neg}Vδ2^{pos} T cells but not statistically significant one.

Importantly, neither Vδ2^{neg} nor Vγ9^{neg}Vδ2^{pos} T-cell percentages nor absolute counts were associated with the other viruses or parasites tested, demonstrating the specificity of CMV influence on both subsets (see Supplementary Figure 3 for percentages; absolute values not shown). As shown in Figure 1C and 1D, among Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells, the percentage of highly differentiated (CD45RA^{pos}CD27^{neg}) cells was higher in CMV+ kidney transplant recipients, whereas the percentage of naive (CD45RA^{pos}CD27^{pos}) cells was higher in CMV− kidney transplant recipients.

Phenotypic Heterogeneity of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T Cells Versus Homogeneity of Vγ9^{pos}Vδ2^{pos} T Cells

To better understand the function and the regulation of γδ T-cell subsets, we compared their expression of natural killer cell receptors, cytotoxic molecules and immune checkpoints in 3 CMV+ kidney transplant recipients. Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells shared the same phenotype, significantly contrasting with that of Vγ9^{pos}Vδ2^{pos} except for granulysin (Figure 2A). A higher proportion of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells than of Vγ9^{pos}Vδ2^{pos} T cells expressed receptors associated with antigen-experienced T cells, such as CD85j, CD158, CD8α, PD-1, and TIM3. Couzi et al [19] previously showed that CMV-experienced Vδ2^{neg} γδ T cells overexpressed CD16, which was also true for Vγ9^{neg}Vδ2^{pos} T cells. By contrast, and consistent with a previous observation of CD161 [20] and KLRG1 [21] down-regulation in memory cells, those markers were expressed in a lower proportion of Vδ2^{neg} or Vγ9^{neg}Vδ2^{pos} T cells than of Vγ9^{pos}Vδ2^{pos} T cells. Likewise, DNAM expression was more predominant in Vγ9^{pos}Vδ2^{pos} T-cell subset. Finally,

Table 1. Characteristics of Transplant Recipients

Characteristic	Transplant Recipients, No./Total (%)[a]		P Value[b]
	CMV Seropositive	CMV Seronegative	
Sex			
Male	36/59 (61)	18/24 (75)	.31
Female	23/59 (39)	6/24 (25)	
Age, mean (SD), y	60 (13)	59 (14)	.92
HLA sensitization			
No	27/59 (46)	13/24 (54)	.54
Yes	32/59 (54)	11/24 (46)	
Number of transplantation			
1	54/59 (92)	21/24 (88)	.68
>1	5/59 (8.5)	3/24 (12)	
rATG induction			
NA	11/59 (19)	4/24 (17)	.56
No	35/59 (59)	13/24 (54)	
Yes	13/59 (22)	7/24 (29)	
Cyclosporine			
No	43/59 (73)	18/24 (75)	.99
Yes	16/59 (27)	6/24 (25)	
Tacrolimus			
No	24/59 (41)	6/24 (25)	.21
Yes	35/59 (59)	18/24 (75)	
MMF			
No	13/59 (22)	9/24 (38)	.17
Yes	46/59 (78)	15/24 (62)	
Corticosteroids			
No	23/59 (39)	9/24 (38)	.99
Yes	36/59 (61)	15/24 (62)	
Azathioprine			
No	56/59 (95)	22/24 (92)	.62
Yes	3/59 (5.1)	2/24 (8.3)	
mTOR inhibitors			
No	47/59 (80)	18/24 (75)	.77
Yes	12/59 (20)	6/24 (25)	
Belatacept			
No	58/59 (98)	24/24 (100)	.99
Yes	1/59 (1.7)	0/24 (0)	
CMV preventive strategy			
NA	11/59 (19)	5/24 (21)	.001
Preemptive	11/59 (19)	13/24 (54)	
Prophylaxis	37/59 (63)	6/24 (25)	
Acute rejection			
No	48/59 (81)	20/24 (83)	.99
Yes	11/59 (19)	4/24 (17)	
Time from transplantation, median (IQR), mo	73 (24–182)	63 (12–187)	.73

Abbreviations: CMV, cytomegalovirus; IQR, interquartile range; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; NA, not available; rATG, rabbit anti-thymocyte globulin; SD, standard deviation.

[a]Data represent no./total (%) of transplant recipients except where otherwise indicated.

[b]P values were obtained with Mann-Whitney or χ^2 tests, as appropriate.

the cytotoxic molecules granzyme B and perforin were more expressed by Vδ2[neg] and Vγ9[neg]Vδ2[pos] than by Vγ9[pos]Vδ2^{pos} T cells.

A t-distributed stochastic neighbor embedding (t-SNE) allowed us to describe more precisely the 3 γδ T-cell subsets. While Vδ2[neg] and Vγ9[neg]Vδ2[pos] T-cell clusters almost always showed similar distributions in the proportion and intensity of each marker tested, Vγ9[pos]Vδ2[pos] T-cell clusters were nearly systematically different (Figure 2B). This was particularly remarkable for CD8α or CD158 expression, which segregated 2 clusters within Vδ2[neg] and Vγ9[neg]Vδ2^{pos} T cells but was uniformly weak
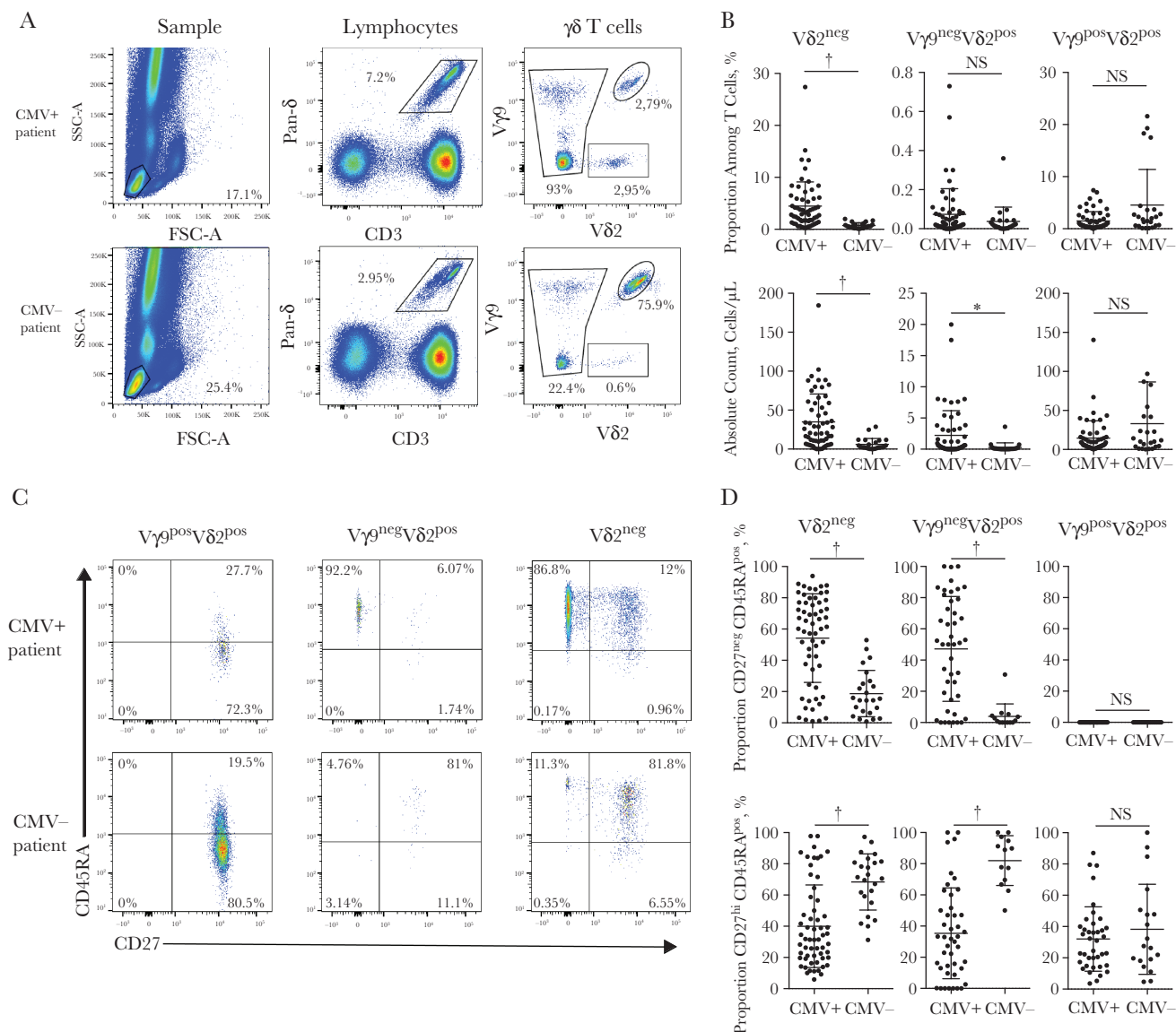
Figure 1. Cytomegalovirus (CMV) serostatus affects the number and the differentiation of Vδ2^neg and Vγ9^neg Vδ2^pos but not of Vγ9^pos Vδ2^pos T cells. Vδ2^neg, Vγ9^neg Vδ2^pos, and Vγ9^pos Vδ2^pos T cells and their expression of CD27 and CD45RA were analyzed for CMV by means of flow cytometry in CMV-seropositive (CMV+; n = 59) and CMV-seronegative (CMV–; n = 24) transplant recipients (CMV serology at the time of whole-blood immunophenotyping). *A,* Whole-blood staining of γδ T-cell subsets in 1 CMV+ and 1 CMV– kidney recipient. *B,* Frequencies among T cells and absolute counts per microliter of blood of each subset in all patients. *C,* Phenotyping of γδ T-cell subsets in 1 CMV+ and 1 CMV– kidney recipient. *D,* Frequencies of TEMRA (TEMRA (T effector memory CD45RA^+)) (CD27^neg CD45RA^pos) and naive (CD27^hi CD45RA^pos) cells in all patients. Each symbol represents an individual donor, and horizontal lines represent means with standard deviations. FSC, forward scatter; SSC, side scatter. *.01 > *P* > .001; †*P* < .001; NS, not significant (*P* > .05) (Mann-Whitney *U* test).

among Vγ9^pos Vδ2^pos T cells. More generally, while several clusters were clearly present within both Vδ2^neg and Vγ9^neg Vδ2^pos T-cell subsets, only 1 largely preponderant cluster with homogeneous expression can be seen within Vγ9^pos Vδ2^pos subsets. In addition, among Vδ2^neg and Vγ9^neg Vδ2^pos T cells, t-SNE analysis revealed coexpression of markers such as CD158 with CD16 or with NKG2C, and granzyme B with perforin and/or granulysin. Conversely, CD16 and NKG2C expressions were mutually exclusive, such as those of DNAM and PD-1.

Direct, Specific, and TCR-Dependent Reactivity of Vγ9^neg Vδ2^pos T Cells Against CMV-Infected Cells

We next tested whether Vγ9^neg Vδ2^pos T cells respond to CMV-infected cells in vitro. Using polyclonal primary γδ T-cell lines, higher proliferation of Vγ9^neg Vδ2^pos and Vδ2^neg T cells was observed when cocultured with CMV-infected fibroblasts than when cocultured with noninfected fibroblasts (Figure 3A). Vγ9^neg Vδ2^pos T cells also specifically responded to CMV-infected fibroblasts by producing IFN-γ (Figure 3B). The percentage of CMV-specific IFN-γ–producing Vγ9^neg Vδ2^pos
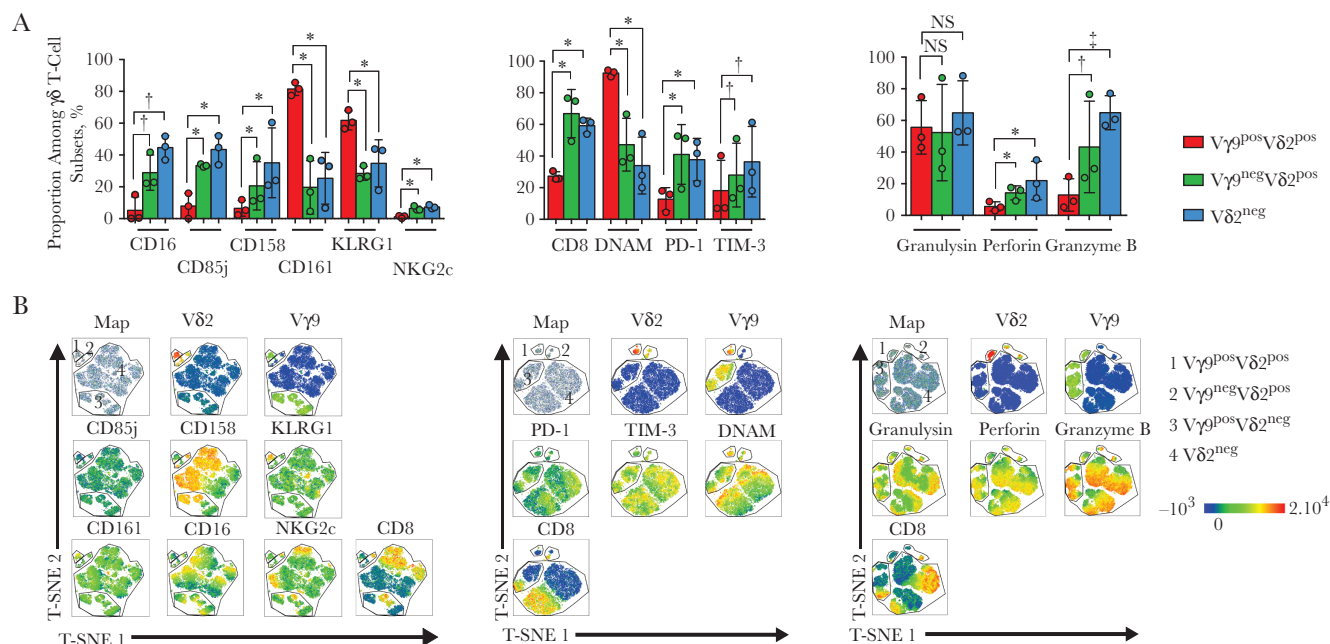
Figure 2. Vδ2^neg and Vγ9^negVδ2^pos T cells share a phenotype that differs from that of Vγ9^posVδ2^pos T cells. Peripheral blood mononuclear cells from 3 cytomegalovirus-seropositive patients were stained with viability marker and monoclonal antibodies directed against CD3, CD8, Vδ2, pan-δ, and the indicated markers. *A*, Frequencies of cells expressing natural killer (NK) receptors, costimulatory and inhibitory receptors, or cytotoxicity markers. Each symbol represents an individual donor; error bars represent means and standard deviations. *.05 > *P* > .01; †.01 > *P* > .001; ‡*P* < .001; NS, not significant (*P* > .05) (paired *t* tests). Abbreviations: DNAM, DNAX accessory molecule; KLRG1, killer cell Lectin-like receptor subfamily G member 1; PD-1, programmed death protein 1; TIM-3, T-cell immunoglobulin and mucin containing protein 3. *B*, t-Distributed stochastic neighbor embedding (t-SNE) plot from 1 representative patient for NK receptors, costimulatory and inhibitory receptors, and cytotoxicity markers among γδ T cells.

T cells was as remarkably high as that of Vδ2^neg γδ T cells (mean [standard deviation], 20.0% [7.7%]; Figure 3B). Highly purified Vγ9^negVδ2^pos T cells secreted similarly high amounts of IFN-γ, as did purified Vδ2^neg γδ T cells from the same patients when cultured with CMV-infected cells (Figure 3C), excluding any indirect activity by other cells present in kidney transplant recipients' PBMCs. For the only kidney transplant recipient with enough Vδ2^posVγ9^neg cells (2.93% of T cells) during CMV infection, we used Vδ2^negVγ9^pos and Vδ2^posVγ9^neg cells purified directly from PBMCs (without preamplification). High activation of these cells by CMV-infected cells was also observed (Supplementary Figure 4). Importantly, fibroblasts infected with HSV-1 or VZV—both belonging to the same beta Herpesviridae family as CMV—were unable to activate IFN-γ production by Vδ2^neg or Vγ9^negVδ2^pos T cells (Figure 3D).

Finally, the addition of a blocking anti–CD3 monoclonal antibody completely inhibited IFN-γ production by Vδ2^neg and Vγ9^negVδ2^pos T cells responding to CMV-infected fibroblasts (Figure 3D) (example from 1 patient shown in Supplementary Figure 5A). This was not due to a decrease of γδ T-cell viability, as assessed by negative viability dye staining (Supplementary Figure 5B).

In Vivo Activation of Vγ9^negVδ2^pos and Vδ2^neg T Cells During the Evolution of CMV Infection

We then prospectively analyzed in vivo the longitudinal activation and expansion of Vγ9^negVδ2^pos T cells during the course of acute

CMV infection from the first positive CMV DNAemia. We showed that Vγ9^negVδ2^pos T cells underwent similar expansion kinetics as Vδ2^neg T cells during acute CMV infection (Figure 4A). Moreover, the evolution of differentiation phenotype over time was strikingly similar in Vδ2^neg and Vγ9^negVδ2^pos T cells, showing an increase in highly differentiated cells and a decrease in naive cells (Figure 4B). A more extensive phenotyping of Vδ2^neg and Vγ9^negVδ2^pos T cells was then performed during and after acute CMV infection and compared with that of CMV+ kidney transplant recipients without posttransplantation infection (inactive infection). This analysis confirmed that both Vδ2^neg and Vγ9^negVδ2^pos T cells were strongly activated in vivo during acute CMV infection and shared common activation markers, such as Ki67, TIM3, DNAM, PD-1, and perforin (Figure 5A). For both subsets, the frequency of Ki67-expressing cells returned to that seen in patients with inactive infection, and the PD-1–positive cells decreased (Figure 5B) after the resolution of CMV DNAemia. Evolution of the other markers was more heterogeneous among patients but similar for both γδ T-cell subsets.

Regarding the expression of the 2 cytotoxic proteins granulysin and granzyme B, an interesting difference in profiles was detected between patients with reinfection and those with primary infection. In the case of reinfection, Vγ9^negVδ2^pos T cells showed an increased percentage of granulysin- and granzyme B–positive cells during infection (Figure 5C), which decreased rapidly after the infection resolved (Figure 5D),
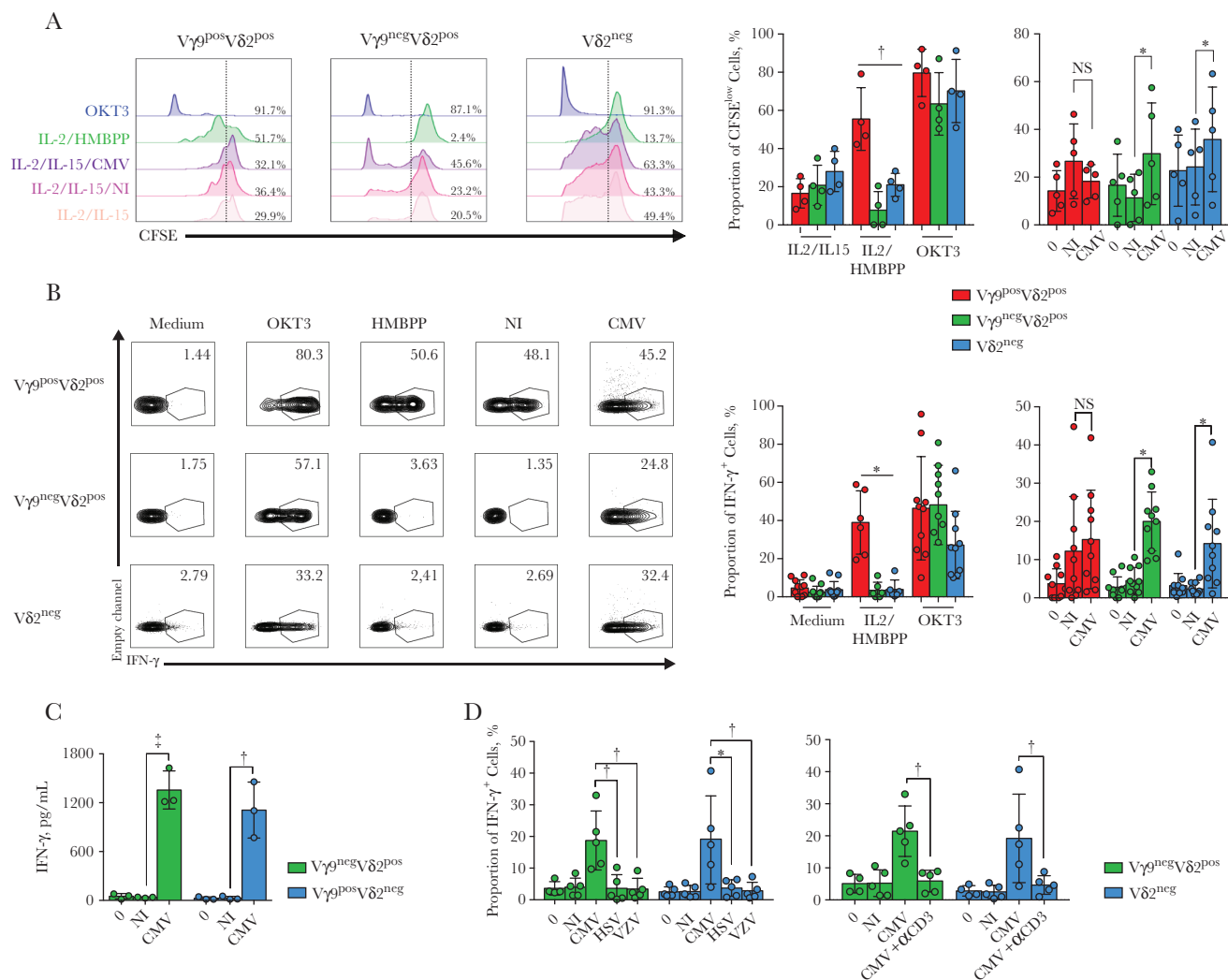
Figure 3. Direct and specific in vitro activation of Vγ9^{neg}Vδ2^{pos} T cells by cytomegalovirus (CMV)–infected cells. *A*, Peripheral blood mononuclear cells (PBMCs) from CMV-seropositive (CMV+) patients were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated either with interleukin 2 (IL-2) and interleukin 15 (IL-15), alone (represented by 0) or in the presence of noninfected (NI) or CMV-infected foreskin fibroblast (FSF) (CMV) (n = 5), with IL-2 and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), or with OKT3 (n = 4). After 7 days, cells were stained with anti-CD3, anti–pan-δ, anti-Vδ2, anti-Vγ9 monoclonal antibodies (mAbs) and the percentage of proliferating cells was determined through CFSE dilution. CFSE profile in 1 patient and mean percentages of proliferating cells. *B*, PBMCs from CMV+ patients were treated in medium alone (represented by 0), with noninfected or CMV-infected FSF (CMV), with OKT3 (n = 10), or with IL-2 and HMBPP (n = 6). After 15 hours, cells were stained with anti-CD3, anti–pan-δ, anti-Vδ2, anti-Vγ9 and anti–interferon (IFN) γ mAbs, and the percentage of IFN-γ–producing cells was determined with flow cytometry. Intracytoplasmic staining of IFN-γ is shown for 1 patient, along with mean percentages of IFN-γ–producing cells. *C*, IFN-γ enzyme-linked immunosorbent assay of highly purified Vγ9^{pos}Vδ2^{neg} or Vγ9^{neg}Vδ2^{pos} T-cell supernatants after 24 hours of culture (medium supplemented with interleukin 18 alone or in the presence of noninfected or CMV-infected FSF). *D*, Intracytoplasmic IFN-γ production by Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells from PBMCs of CMV+ patients cultured for 15 hours in medium alone; or with noninfected, CMV-infected FSF with or without blocking anti-CD3 mAb (10 μg/mL) and herpes simplex virus (HSV)– or varicella-zoster virus (VZV)–infected FSF (multiplicity of infection for each virus, 0.1) (n = 5). Each symbol in *A, B,* and *D* represents an individual donor, and each symbol in *C* represents the mean of duplicate cultures from an individual donor; error bars represent means and standard deviations. *.05 > *P* > .01; †.01 > *P* > .001; ‡*P* < .001; NS, not significant (*P* > .05) (paired *t* test).

whereas Vδ2^{neg} γδ T cells showed a delayed increase in the percentage of cells expressing granulysin. By contrast, during primary infection, Vγ9^{neg}Vδ2^{pos} and Vδ2^{neg} γδ T cells displayed similarly late kinetics of granulysin and granzyme B expression, characterized by a significant increase in positive cells only after the infection was resolved (Figure 5C and 5D). In summary, circulating Vγ9^{neg}Vδ2^{pos} T cells are activated and expand during CMV infection, with a more rapid and transient cytotoxic potential exhibited during reinfection.

Correlation of Vγ9^{neg}Vδ2^{pos} but Not Vδ2^{neg} γδ T Cells With CMV Infection Severity

To analyze the influence of CMV infection clinical intensity on Vγ9^{neg}Vδ2^{pos} T-cell activation, we compared kidney transplant recipients with moderate versus severe disease (as defined in the Supplementary Methods). The maximum percentages of Vγ9^{neg}Vδ2^{pos} T cells during the course of infection were higher in kidney transplant recipients with severe disease (Figure 6A) and were positively correlated with CMV
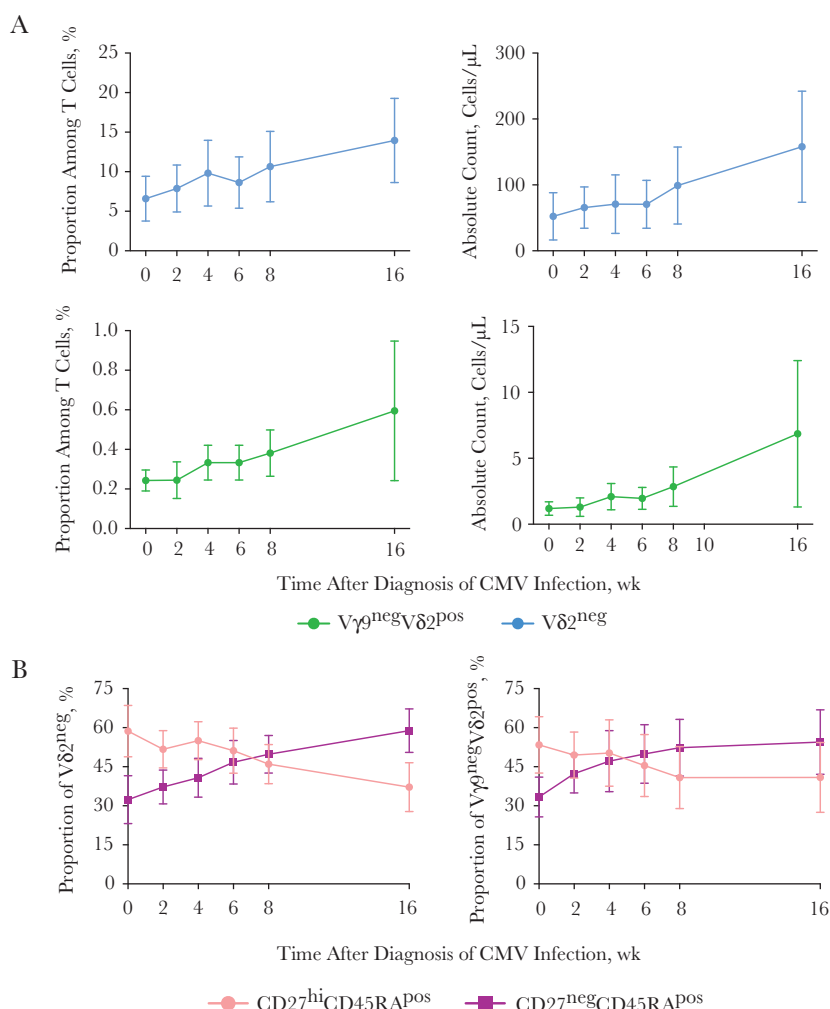
Figure 4. Kinetics of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T-cell response during cytomegalovirus (CMV) infection. *A*, Frequencies among T cells and absolute counts of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells were evaluated longitudinally in 6 patients with acute CMV infection from the diagnosis of CMV replication. Assessment of CMV DNAemia (whole-blood quantitative nucleic acid testing) was performed at the same time as immunophenotyping. *B*, Frequencies of highly differentiated (CD27^{neg}CD45RA^{pos}) and naive (CD27^{hi}CD45RA^{pos}) cells among Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells from 6 patients with CMV infection were analyzed longitudinally from the beginning of CMV detection. Each symbol represents the mean of values for all donors, and error bars represent standard errors of the mean.

DNAemia duration (from the first positive CMV polymerase chain reaction to negativation) (Figure 6B). Patients with primary infection and reinfection were equally divided with regard to disease severity. It is noteworthy that these clinical parameters had no influence on Vδ2^{neg} γδ T-cell percentages. These observations indicate that expansion of Vγ9^{neg}Vδ2^{pos} T cells, unlike Vδ2^{neg} γδ T cells, is strongly driven by the severity of CMV infection.

These parameters were then analyzed in our retrospective cohort of 83 patients. In the 26 kidney transplant recipients in whom posttransplantation CMV DNAemia had developed, Vγ9^{neg}Vδ2^{pos} T-cell frequencies ($P < .001$; Figure 6C), absolute counts ($P = .02$; not shown), and differentiation (percentage of CD45RA^{pos}CD27^{neg} cells; $P = .03$; Figure 6C) were conspicuously higher in patients with CMV disease than in those without disease. Here again no difference was observed

for Vδ2^{neg} γδ T cells. Moreover, the frequency and the absolute count of Vγ9^{neg}Vδ2^{pos} (vs Vδ2^{neg} γδ) T cells was correlated with the duration of CMV DNAemia (Figure 6D; absolute count not shown). CMV disease (Fisher test, $P = .1$) and the duration of CMV DNAemia (Supplementary Figure 6) were not different for kidney transplant recipients with primary infection or reinfection. Altogether, these results suggest that Vγ9^{neg}Vδ2^{pos} T cells are more prone to expand during the acute phase than to differentiate and be maintained (and/or to recirculate) in the long term when CMV infection is severe.

DISCUSSION

In the present study, we provide an extended analysis of Vγ9^{neg}Vδ2^{pos} T cells in a substantial cohort of 83 kidney transplant recipients, demonstrating their expansion and differentiation during the course of CMV infection, their direct and
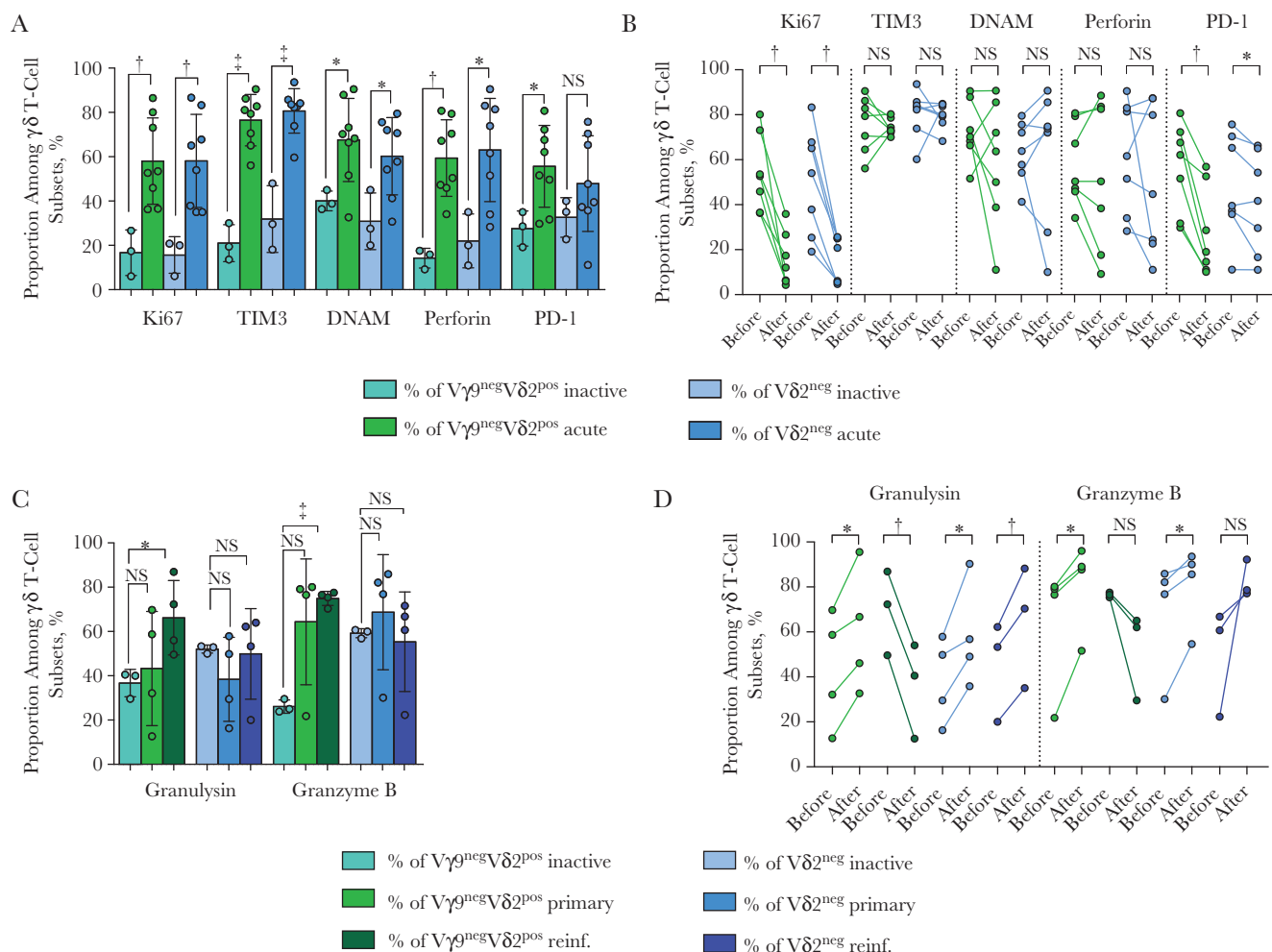
Figure 5. Phenotypic evolution of Vδ2^neg and Vγ9^neg Vδ2^pos T cells during cytomegalovirus (CMV) primary infection and reinfection. Peripheral blood mononuclear cells from CMV-seropositive patients without posttransplantation CMV infection (inactive) or from patients with posttransplantation CMV reinfection or primary infection (all together labeled "acute" in *A* and *B*) were stained with monoclonal antibodies directed against the indicated markers and against CD3, pan-δ, Vδ2, and Vγ9 and analyzed by means flow cytometry. *A, C,* Comparison of the percentages of Vδ2^neg and Vγ9^neg Vδ2^pos T cells expressing the indicated markers during inactive infection versus during the peak of acute infection (*A,* reinfection and primary infection together; *C,* reinfection and primary infection analyzed separately). Each symbol represents an individual donor; error bars indicate means and standard deviations. *B, D,* Comparison of marker expressions during the peak of infection (after) and after CMV DNAemia negativation (after) in the same patients. Each symbol represents an individual donor. *.05 > *P* > .01; †.01 > *P* > .001; ‡*P* < .001; NA, not significant (*P* > .05) (unpaired [*A, C*] or paired [*B, D*] *t* tests). Abbreviations: DNAM, DNAX accessory molecule; PD-1, programmed death protein 1; TIM-3, T-cell immunoglobulin and mucin containing protein 3.

specific reactivity to CMV-infected fibroblasts and their remarkable and independent association with infection severity.

The analysis of this large cohort of kidney transplant recipients gave robust evidence that Vγ9^neg Vδ2^pos T cells are closer to Vδ2^neg γδ than to Vγ9^pos Vδ2^pos T cells, regarding their expansion and differentiation in response to CMV. We provided here additional arguments to define Vγ9^pos Vδ2^pos T cells as an innatelike T cell-subset with an homogeneously high expression of innatelike markers [22] and a very low level of adaptive inhibitory immune checkpoints, consistent with their already described semi-invariant TCR repertoire [12].

Conversely, Vγ9^neg Vδ2^pos T cells expressed the adaptive T-cell features that were previously shown for Vδ2^neg γδ T cells [7, 11, 23], as illustrated by a preponderant naive

phenotype in CMV− subjects and a late-differentiated phenotype in CMV+ ones. Here, an extensive phenotyping provided important information about heterogeneous expression levels of both activating and inhibitory receptors, suggesting a diversification of this population. This diversity could be due to sequential stimulations by recurrent CMV replication episodes, either clinical or subclinical, which could be particularly frequent in the context of immunosuppressive therapy, as well as in response to a large diversity of antigens. Altogether, those characteristics are consistent with the adaptive status of Vγ9^neg Vδ2^pos T cells, also demonstrated by the widely diversified TCR repertoire they express [12] and the clonal expansion they undergo during CMV infection [13, 24].
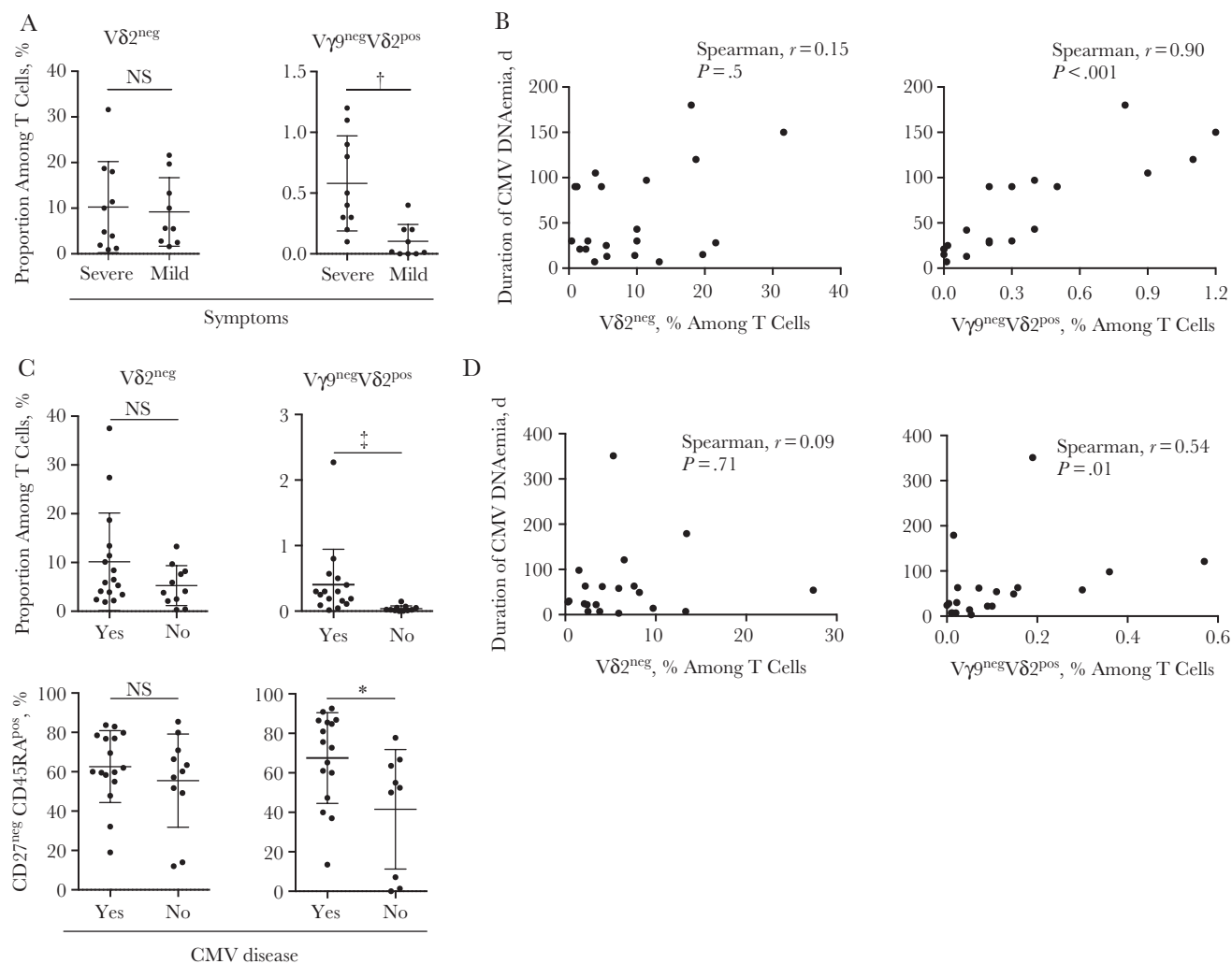
Figure 6. Vγ9^{neg}Vδ2^{pos} T cells are better correlated with cytomegalovirus (CMV) infection severity than Vδ2^{neg} gδ. *A*, Frequencies of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells were evaluated in kidney transplant recipients during acute CMV DNAemia with mild (n = 9) or severe (n = 10) symptoms. *B*, Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T-cell percentages during acute CMV infection were plotted against CMV DNAemia duration (days between the first positive CMV DNAemia and negativation). *C*, Frequencies and TEMRA (T effector memory CD45RA^{+}) (CD45RA^{pos}CD27^{neg}) phenotype of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells were evaluated in kidney transplant recipients with CMV DNAemia after transplantation associated (n = 16) or not associated (n = 11) with disease. *D*, Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T-cell percentages during CMV infection after transplantation were plotted against CMV DNAemia duration. Each symbol represents an individual donor; horizontal lines in *A* and *C* represent means and standard deviations. *.05 > *P* > .01; †.01 > *P* > .001; ‡*P* < .001; NS, not significant, *P* > .05 (Mann-Whitney *U* test). Correlation coefficients were determined with the Spearman test (*B, D*).

An important remaining question was the direct reactivity of this Vγ9^{neg}Vδ2^{pos} T-cell subset to CMV-infected cells. We show here that Vγ9^{neg}Vδ2^{pos} T cells exhibited specific and TCR-dependent reactivity to CMV-infected cells. We also provide the first demonstration of a direct ex vivo reactivity to CMV-infected cells by polyclonal Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells, which was previously observed mainly using γδ Tcell clones or cell lines [5, 6]. This reactivity led to the proliferation of Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells and to the production of IFN-γ, a well-known antiviral cytokine able to inhibit CMV DNA replication and viral protein expression and to promote viral antigen presentation to enhance CD8 T-cell response [25].

Importantly, no reactivity against VZV- or HSV-infected cells could be observed in vitro, nor expansion of γδ T cells

in kidney transplant recipients infected with these viruses or other infectious pathogens, as reported elsewhere [5, 7]. CMV is therefore a unique herpesvirus able to mobilize Vδ2^{neg} and Vγ9^{neg}Vδ2^{pos} T cells, raising the important and not fully resolved issue of the molecular determinants of this striking specificity. This specificity is unlikely to be explained by γδ TCR recognition of CMV-encoded determinants, as it has been previously demonstrated that TCRs from CMV-specific Vδ2^{neg} γδ T cells recognize stress-induced self-antigens expressed by both CMV-infected cells and tumor cells (free of CMV) [5, 26, 27]. Further studies are required to clarify which stress pathways inducing γδ T-cell reactivity are elicited by CMV or cell transformation and not by HSV or VZV.

Even if $V\gamma9^{neg}V\delta2^{pos}$ and $V\delta2^{neg}$ T cells exhibited similar reactivity to CMV, they also have nonredundant functions, as their behaviors regarding clinical evolution of CMV infection were not identical. Both subsets are involved during CMV primary infection and reinfection, as depicted by expansion, increased expression of activation markers, and switch from a predominant naive to a TEMRA phenotype. However, the kinetics of $V\gamma9^{neg}V\delta2^{pos}$ and $V\delta2^{neg}$ $\gamma\delta$ T-cell responses to CMV showed some differences, with more rapid and transient expression of granulysin and granzyme B only on $V\gamma9^{neg}V\delta2^{pos}$ during reinfection. Either $V\gamma9^{neg}V\delta2^{pos}$ T cells are promptly negatively regulated and/or they are quickly recruited in infected tissues, leading to a decrease in peripheral blood in case of reinfection. Finally, the nonredundant function of $V\delta2^{neg}$ $\gamma\delta$ T and $V\gamma9^{neg}V\delta2^{pos}$ T cells is especially evidenced by the significant association between higher $V\gamma9^{neg}V\delta2^{pos}$ T-cell expansion and severe CMV infection, both during the acute phase and in the long term.

Consequently, we could suggest a hierarchical recruitment of different $\gamma\delta$ T-cell subsets in immunocompromised host during CMV infection [4, 6, 24, 28]. In mild to moderate infection, $V\delta2^{neg}$ $\gamma\delta$ T cells could act as a first-line defense, promptly recruited and circulating all over the body to control systemic infection. However, when CMV infection is uncontrolled or more life-threatening, $V\gamma9^{neg}V\delta2^{pos}$ T cells could be recruited as an additional and elective contingent, representing a sort of "tactical reserve" and, to our knowledge, the only T-cell subset with such a specificity. In conclusion, our results help extend the functional family of CMV-reactive T cells, providing thus new insights on this unique subset of human $V\gamma9^{neg}V\delta2^{pos}$ T cells, with unanticipated properties and interest as a prognosis immune marker of CMV infection severity, which could help CMV management in immunocompromised patients.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Single-cell RNA sequencing unveils the shared and the distinct cytotoxic hallmarks of human TCRVδ1 and TCRVδ2 γδ T lymphocytes

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γδ T lymphocytes represent ~1% of human peripheral blood mononuclear cells and even more cells in most tissues of vertebrates. Although they have important anticancer functions, most current single-cell RNA sequencing (scRNA-seq) studies do not identify γδ T lymphocytes because their transcriptomes at the single-cell level are unknown. Here we show that high-resolution clustering of large scRNA-seq datasets and a combination of gene signatures allow the specific detection of human γδ T lymphocytes and identification of their T cell receptor (TCR)Vδ1 and TCRVδ2 subsets in large datasets from complex cell mixtures. In t-distributed stochastic neighbor embedding plots from blood and tumor samples, the few γδ T lymphocytes appear collectively embedded between cytotoxic CD8 T and NK cells. Their TCRVδ1 and TCRVδ2 subsets form close yet distinct subclusters, respectively neighboring NK and CD8 T cells because of expression of shared and distinct cytotoxic maturation genes. Similar pseudotime maturation trajectories of TCRVδ1 and TCRVδ2 γδ T lymphocytes were discovered, unveiling in both subsets an unattended pool of terminally differentiated effector memory cells with preserved proliferative capacity, a finding confirmed by in vitro proliferation assays. Overall, the single-cell transcriptomes of thousands of individual γδ T lymphocytes from different CMV+ and CMV− donors reflect cytotoxic maturation stages driven by the immunological history of donors. This landmark study establishes the rationale for identification, subtyping, and deep characterization of human γδ T lymphocytes in further scRNA-seq studies of complex tissues in physiological and disease conditions.

γδ T lymphocyte | transcriptome | single-cell RNA-sequencing | human immunology | cancer

Single-cell level mRNA-sequencing (scRNA-seq) of heterogeneous cell populations has become the reference tool for establishing cellular lineages and composition of tissues from the human body (1). In addition, the development of novel and open-source computational tools for processing scRNA-seq datasets enables delineation of both broad and subtle differences in rare cell subsets present in complex mixtures, such as peripheral blood mononuclear cells (PBMC) (2, 3). Such developments are expected to build more knowledge about the cellular composition and states composing tumors. Recent scRNA-seq analyses in melanoma and colorectal cancer evidenced the various tumor microenvironments and exhaustion patterns of the tumor-infiltrating lymphocytes (4). Most of such

studies currently focus on the cytotoxic CD8 T lymphocytes, which represent the main target of immune checkpoint blockade therapies, and are readily detected by their scRNA-seq profile. Other subsets of cytolytic T cells are also critical in this therapeutic perspective; however, currently they have never been characterized by scRNA-seq and are therefore missing from all current tumor microenvironments mapped by these technologies.

Human γδ T lymphocytes represent a peculiar lymphoid cell subset displaying hallmarks of both innate and adaptive immunity, and reacting to microbial pathogens and malignancies (5). These CD4− CD8− T cells express a somatically rearranged

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Significance

None of the single-cell RNA sequencing (scRNA-seq) studies published so far convincingly identified human γδ T lymphocytes despite their anticancer functions. To address this, we here report scRNA-seq of γδ T lymphocytes purified from human blood, and a signature identifying γδ T cells. The single-cell transcriptomes of TCRVδ1 and TCRVδ2 lymphocytes are intermediates resembing those of NK cells and T CD8, respectively, they reflect their respective maturation stage and their pseudotime maturation trajectory unveils terminally differentiated cells with mitotic capacity. In human cancers, the γδ TIL, mostly expressing TCRVδ1, appear fewer in tumors than in blood and not correlated to αβ TIL abundance. This report is a landmark resource for future studies of γδ T lymphocytes at the single-cell level.

T cell receptor (TCR), as well as cytotoxicity receptors more typically expressed by NK cells. The diversity of γ- and δ-encoded TCR relies on VJ rearrangements of the *TRG* locus at 7p14 and VDJ rearrangements of the *TRD* locus at 14q11.2, respectively. In the human genome, the *TRD* locus contains three variable genes (*TRDV1–3*), three diversity genes (*TRDD1–3*), four joining genes (*TRDJ1–4*) and one constant *TRDC* gene, which rearrange to encode a TCRδ chain. The *TRG* locus that rearrange to encode a TCRγ chain contains 14 variable genes, of which only 6 are functional (*TRGV2–5*, *TRGV8*, and *TRGV9*), five joining genes (*TRGJ1, J2, JP, JP1, JP2*), and two constant genes (*TRGC1, TRGC2*). Despite this low number of TCR-encoding gene segments, the human TCR repertoire of γδ T cells is almost as diversified as that of αβ T lymphocytes. In addition, the repertoire of γδ TCR expressed at the cell surface of γδ T cells is biased according to their tissue localization. *TRGC1* encodes for the γ-constant region of the cell surface TCRVγ9Vδ2 expressed by the most abundant γδ T lymphocytes in human adult blood, a subset of γδ T cells detecting microbial and tumoral metabolites called phosphoantigens (PAgs) (6) associated to the non-HLA butyrophilin-3 molecule (7). In contrast, *TRGC2* encodes for the γ-constant region shared by all of the cell surface TCRVγ(non-9)Vδ2-, TCRVδ1-, and TCRVδ3-expressing γδ T cells, which are generally less frequent than Vγ9Vδ2 cells in adult blood but predominate in other tissues, and recognize different antigens. The TCRVδ1+ lymphocytes represent the prominent non-Vγ9Vδ2 γδ T cell subset and are mainly located in adult skin, lung, intestine, and colon epithelia, where they recognize antigens from virally infected and cancer cells (8).

Similarly, non-(TCRVδ2) γδ T cells are induced by environmental cytomegalovirus (CMV) (9), are associated with a reduced risk of cancer in immunosuppressed patients (10), and some of these lymphocytes recognize the endothelial protein C receptor overexpressed by carcinoma cells (11). TCRVδ3 cells represent a rarer γδ T cell subset in blood, and some TCRVγ8Vδ3 T lymphocytes recognize Annexin A2 from stressed and cancer cells (12). Hence, all of the TCR-based subsets of γδ T cells might participate to antitumor immunity, although by coreceptors and functions depending on the stage of maturation reached by these T lymphocytes. Upon antigenic stimulation, the γδ T lymphocytes successively mature from naïve (CD27+, CD62L+ CCR7+, CD45RA+) cells to central memory cells (CD27+, CD62L+ CCR7+, CD45RA−) with strong proliferative and low effector function. Upon further Ag stimulation, they may further mature into effector memory cells (CD27−, CD45RA− lymphocytes producing either IFN-γ or granzyme/perforin), and finally drive to terminally differentiated CD45RA-expressing terminally differentiated effector memory (TEMRA) cells (CD45RA+ CD16+) essentially mediating the ADCC-type of cytotoxic function. This maturation pathway, spanning from naïve to TEMRA cells, was identified in TCRVδ2+ γδ T lymphocytes, whose TCR activation precedes and progressively drives expression of cytotoxicity receptors shared with NK cells (13–16). Other γδ T cells, such as the CMV-reactive γδ T lymphocytes, also predominantly display a TEMRA and CD16+ phenotype with adaptive-like response to CMV (17–20). Most TCRVδ1+ γδ T cells may expand in a CDR3-independent (21), but AKT/γc cytokine-driven fashion (22), and progressively express cytolysis-inhibiting as well as natural cytotoxicity receptors. Hence whatever the TCR subset, this blend of innate and adaptive skills makes all γδ T lymphocytes with NK-like functions attractive candidates for controlling viral infections (23) and cancer (5, 24). Given the recent developments in adoptive γδ T cell therapies of cancer (25), it is important to know whether all subsets of γδ T cells mature similarly, but this remains unclear so far.

Furthermore, for cancer therapy, determining the rate of tumor-infiltrating γδ lymphocytes (γδ TIL) from any tumor biopsy is critical. CIBERSORT is a recent algorithm deconvoluting the composition of TILs from microarrays of cancer biopsies (26), and its use to analyze 19,000 tumors concluded that rate of

γδ TILs positively correlates with good outcome (27). Although encouraging, such results suffered of poor learning from too few (only two) γδ T cell transcriptomes, however, as CIBERSORT identifies erroneously most of CD8 T, NK cells, and γδ T lymphocytes (28). This problem reflects the massive gene multi-collinearity of transcriptomes from these three closely related cell types (29), suggesting that deeper learning from many more γδ T cell transcriptomes is necessary. In addition to unfaithfully identifying γδ T cells as a whole, determining their subsets defined by cell surface TCR and stage of maturation is currently out of reach for the same reasons. Thus, a decisive milestone would be the straightforward identification of γδ T lymphocytes from scRNA-seq data. Such an achievement could allow us to determine their presence, their TCR, maturation stage, and activation/exhaustion status in the tumor microenvironment of a large panel of human cancers. Nevertheless, in this aim it remains necessary to identify γδ T lymphocytes from nonmalignant reference tissue samples, such as PBMC from healthy individuals. Because these lymphocytes are unfrequent (1–5% of PBMC) and their transcriptomes share cytotoxic hallmarks of both CD8 T and NK cells (29), they are not identified as such in current *t*-distributed stochastic neighbor embedding (t-SNE) plots of scRNA-seq datasets, which posit each cell within its "alikes" to construct clusters. Hence, in most t-SNE published so far, γδ T lymphocytes are generally embedded within larger clusters of CD8 T and NK cells without being correctly identified, despite their biological importance.

To overcome these problems, herein we produced scRNA-seq transcriptomes of blood γδ T lymphocytes of TCRVδ1+ and TCRVδ2+ subsets purified from three healthy donors, and depicted their respective gene-expression profiles at this high-definition level. These data represent a valuable resource for further studies delineating the landscape of γδ T lymphocytes in human physiology and disease.

Results

High Clustering Levels and High-Resolution t-SNE Plots of Large Datasets Are Required to Spot γδ T Lymphocytes from scRNA-Seq of Complex Tissues.

A CMV+ healthy donor #1 comprising 2.67% of γδ T lymphocytes in PBMC was selected for this study; ~1,000 PBMC isolated from this donor were analyzed by scRNA-seq by using the 10x Genomics single-cell 3′ V2 chemistry, followed by sequencing of 96 million reads per library. After alignment on the GRCh38 transcriptome, a total of 15,309 genes were detected with an average of 120,000 reads for 1,314 unique genes per cell. Because γδ T cells represent only ~1–5% of PBMC and cell resolution of scRNA-seq analyses increases with larger datasets, we also downloaded from 10x Genomics two additional scRNA-seq datasets produced by the same technology of 4k and 8k PBMC derived from one healthy donor (https://support.10xgenomics.com/single-cell-gene-expression/datasets). These latter datasets represent 4,340 PBMC with an average of 87,000 reads for 1,235 genes per cell (4k dataset), and 8,381 PBMC with an average of 95,000 reads for 1,297 genes per cell (8k dataset). These three datasets, totaling ~13,000 PBMC, were integrated, aligned, log-normalized using the "Seurat alignment" workflow (2), and quality-controlled (QC) for number of genes per cell and mitochondrial gene content. Twelve thousand single cells passed the QC filter, with an average of 1,320 unique genes detected per cell. Unsupervised analysis of this 12k PBMC dataset identified 11 statistically significant principal components used by the Seurat package's functions FindClusters and RunTSNE to partition the dataset into several clusters of cells. The nonlinear dimensionality reduction of this dataset by t-SNE (30) using a low (i.e., 0.6) resolution of this graph-based clustering produced eight clusters of cells visualized on a t-SNE map (Fig. 1*A*). The mRNA biomarkers identified these clusters as those of classic and intermediate monocytes (e.g., *LYZ, S100A9, CD14*), dendritic cells (DC) (*LYZ, S100A9, FCER1A*), intermediate monocytes (*CD14−, LYZ, S100A9, FCGR3A*), B cells (*CD79A,*
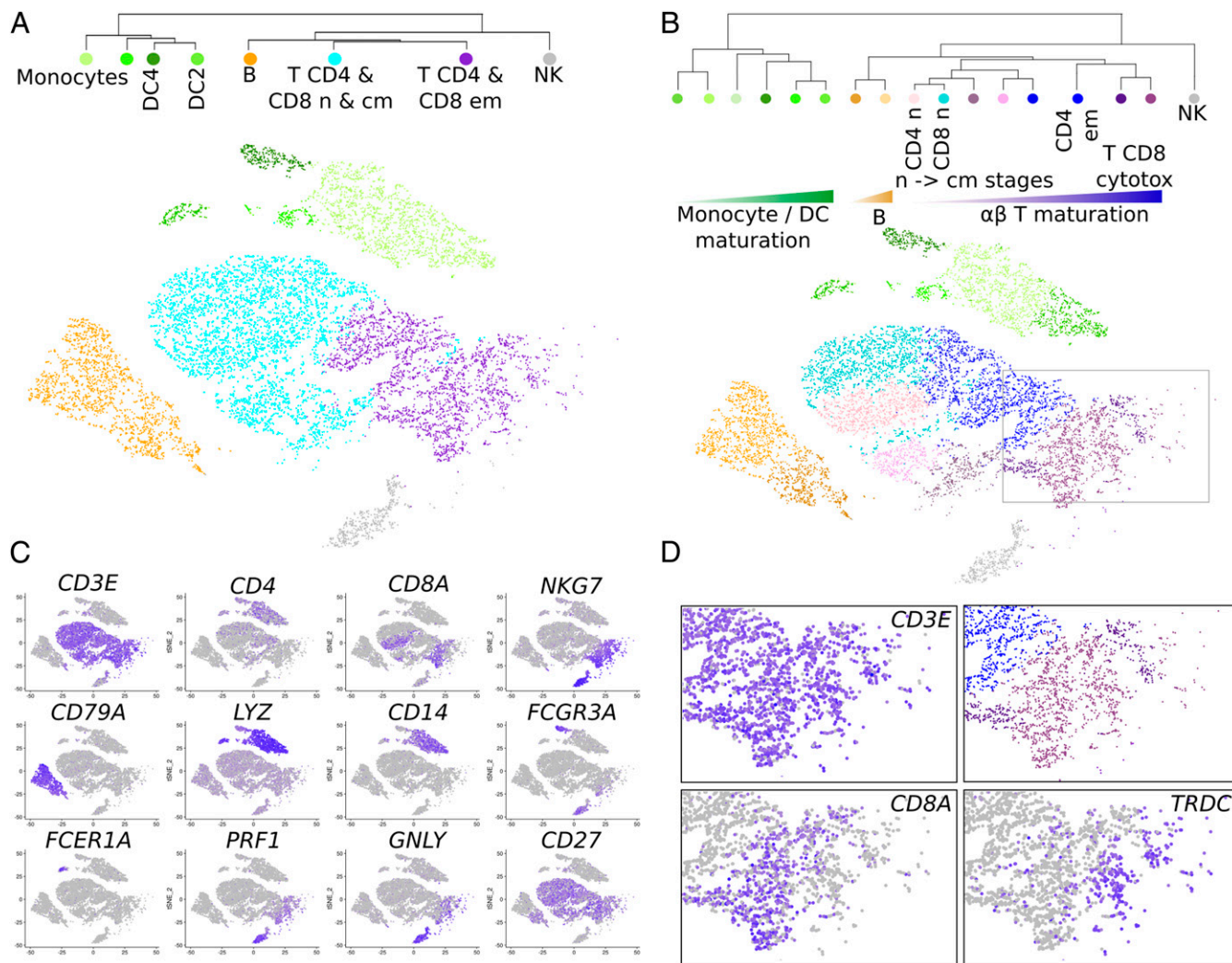
Fig. 1. Visualization of γδ T lymphocytes from PBMC scRNA-seq requires high resolution and clustering depth t-SNE plots of large datasets. (*A*) Unsupervised hierarchical clustering and correspondingly colored t-SNE plot at low resolution (granularity 0.6) of 12k PBMC partitions eight clusters lacking a γδ T lymphocyte cluster. (*B*) Expression levels of the specified biomarker genes (purple) on the same t-SNE plot. (*C*) Unsupervised hierarchical clustering and correspondingly colored t-SNE plot at high resolution (granularity 1.2) of the same dataset partition 17 clusters, of which one (boxed area) comprises cytotoxic CD8 T and γδ T cells. (*D*) Zoom-in of the boxed region (*Top Right*) with expression levels (purple) of the specified genes.

CD79B, *CD19*), naïve and central memory CD4 and CD8 T cells (*CD3E*, *CD4* or *CD8A*, *SELL*, *CD27*), effector memory CD4 and CD8 T cells (*CD3E*, *CD4* or *CD8A*, *CD27*, *PRF1*, *GNLY*), and NK cells (*CD3E*-negative*NKG7* cells) (Fig. 1*B*). This classification was consistent with hematopoietic differentiation and the previously published t-SNE plots of PBMC scRNA-seq. (31–33).

However, despite showing that transcriptomic signatures of blood T lymphocytes primarily reflect maturation stage rather than merely CD4 or CD8 lineages, this t-SNE plot did not evidence a γδ T cell cluster. To delineate such a cluster, expected to encompass infrequent *CD3+CD4−CD8−* cells expressing *TRDC* that encodes for the unique constant region of the TCRδ chain, the same dataset was analyzed as above and then visualized by t-SNE plots with a higher (i.e., 1.2) resolution. This partitioned 17 clusters that did not subdivide the previous NK cell cluster, but created six clusters of monocytes and DCs, two B cell clusters, and eight T cell clusters discriminating CD4 from CD8 T cells and their maturation stages (e.g., *SELL*, *CD27*, *ITGAL*, *IL7R*) (Fig. 1*C*). In these settings, the most mature cytotoxic T cell cluster (defined by *FCGR3A* and *GNLY*) included both *CD3E+CD8A+* T cells and *CD3E+CD4−CD8A−* cells that

expressed the TCRδ constant region-encoding segment *TRDC*, presumably corresponding to γδ T lymphocytes (Fig. 1*D*). Thus, high-resolution clustering of large scRNA-seq datasets and selected sets of genes may allow for the spotting of γδ T lymphocytes in complex cell mixtures from heterogeneous tissues, such as a bulk PBMC.

A Gene Signature to Detect Human γδ T Lymphocytes in Complex Cell Mixtures. In peripheral blood of adults, γδ T lymphocytes usually represent 1–4% of PBMC and present a [CD3+TCRγδ+CD4−CD8−] cell surface phenotype. Nevertheless, here the corresponding [CD3E+TRDC+CD4−CD8A−] gene-expression criterion identified $n = 862$ cells (7.2%) of the 12k PBMC dataset (Fig. 2*A*). This probably overestimated γδ T lymphocytes, as $n = 487$ of these cells were likely false-positives dispersed in the myeloid, B cells, NK cells, CD4, and CD8 T lymphocytes clusters. Consistent with the transcriptome hallmarks of bulk γδ T lymphocytes (29), however, $n = 375$ γδ T cell candidates were mapped between the cytotoxic CD8 T and NK cell clusters. Nevertheless, the single-cell transcriptomes of cytotoxic CD8 T and NK cells perplexed the delineation of γδ T lymphocytes. On the one hand,

Fig. 2. Gene signatures identifying γδ T lymphocytes. (*A*) In the 12k PBMC dataset, *n* = 862 (7%) PBMC express the CD3E and TRDC but not CD4 and not CD8A genes (red), including *n* = 375 cells mapped between the cytotoxic CD8 T and NK cell clusters as expected for γδ T lymphocytes. (*B*) In the same dataset, *n* = 515 (4%) PBMC are detected by the combined signature score (*SI Appendix, Material and Methods*), including *n* = 418 cells mapped between the cytotoxic CD8 T and NK cell clusters. (*C*) In the dataset integrating both 12k PBMC and 1,333 purified γδ T lymphocytes from CMV[+] donor #1, *n* = 1,683 cells are identified as γδ T lymphocytes. These include the above 418 PBMC-derived γδ T embedded in the *n* = 1,265 purified γδ T lymphocytes. (*D*) ROC curves for γδ T cell detection in the 13k dataset, using the genes depicted in *A*, or the signature combination used for *B*.

frequent single cells of the NK cluster from this PBMC dataset (*SI Appendix*, Fig. S1*A*) and from other datasets of purified NK cells (34) (*SI Appendix*, Table S1) strongly express the γδ TCR-encoding gene segments *TRDC*, and more weakly the *TRGC1* and *TRGC2* segments. On the other hand, cytotoxic CD8 T lymphocytes frequently and strongly express *TRGC2*, but weakly *TRDC* and *TRGC1* (*SI Appendix*, Fig. S1*B*).

Furthermore, blood γδ T lymphocytes comprise variable rates of cells with TCRVδ2 encoded by the *TRGC1* and *TRDC* gene segments, and of cells with TCRVδ1 encoded by the *TRGC2* and *TRDC* gene segments, although both subsets express some level of each other's *TRGC1* and *TRGC2* (*SI Appendix*, Fig. S1*B*). Therefore, to identify γδ T lymphocytes exhaustively and without NK and T CD8 false-positives, we designed a more performant γδ signature combining two gene sets (*SI Appendix, Material and Methods*) that was scored for each single cell and visualized in the t-SNE by Single-Cell Signature Explorer (35). In the 12k PBMC dataset, this γδ signature score was >0.35 for *n* = 515 cells, including 418 (3.4% of PBMC) cells mapped between the T CD8 and NK cell clusters (Fig. 2*B*). To validate this γδ signature, highly pure (>99%) (*SI Appendix, Material and Methods*) γδ T lymphocytes were positively sorted by anti-TCRVδ1 and anti-TCRVδ2 mAbs from the same CMV[+] blood donor #1 as for the above PBMC. After scRNA-seq, pre-processing, and QC filtering as above, the pooled transcriptomes of *n* = 1,333 of 1,529 purified TCRγδ[+] T lymphocytes were obtained, with a mean of 263k reads for 12,412 unique genes per

cell. After integration, alignment, and processing with the above 12k PBMC dataset, the resulting 13k dataset was visualized by high-resolution t-SNE plot. Most (*n* = 1,265 of 1,333, 95%) purified γδ T lymphocytes colocalized with the 418 PBMC between T CD8 and NK, and (*n* = 1,020 of 1,265, 80%) were positive for the γδ signature (Fig. 2*C*). When the performances of the [*CD3E*^{+}*TRDC*[+]*CD4*^{−}*CD8A*^{−}] gene set and the γδ signature were compared for detecting γδ T lymphocytes within the 13k dataset, their ROC curves showed that the γδ signature was superior (Fig. 2*D*). These and additional results (see below) validated the identification of γδ T lymphocytes by the combination signature.

Using the same processing and combination signature, the rate of γδ T cells was determined in additional scRNA-seq datasets of PBMC from healthy donors and tumor biopsies from cancer patients. This approach identified 3.2% and 5% of γδ T lymphocytes in PBMC from two other healthy donors (Fig. 3 *A* and *B*). Although TILs are less abundant in tumors, some γδ TILs could nevertheless be detected in such samples. Among *n* = 2,933 total blood cells from a relapsed chronic lymphocytic leukemia (CLL) patient, *n* = 23 TILs, including *n* = 3 γδ T lymphocytes, were detected (Fig. 3*C*). In the tumor biopsy from a freshly diagnosed follicular lymphoma (FL) (36), *n* = 13 γδ T lymphocytes of 472 TILs (3.3%) were detected (Fig. 3*D* and *SI Appendix*, Fig. S2), consistent with the 5% γδ TILs rate reported in this malignancy (27, 28, 37). In a nonsmall cell lung cancer (NSCLC) tumor sample comprising 252 TILs, only *n* = 3 γδ T lymphocytes (1.2% of TIL) were detected, while *n* = 10 γδ T of

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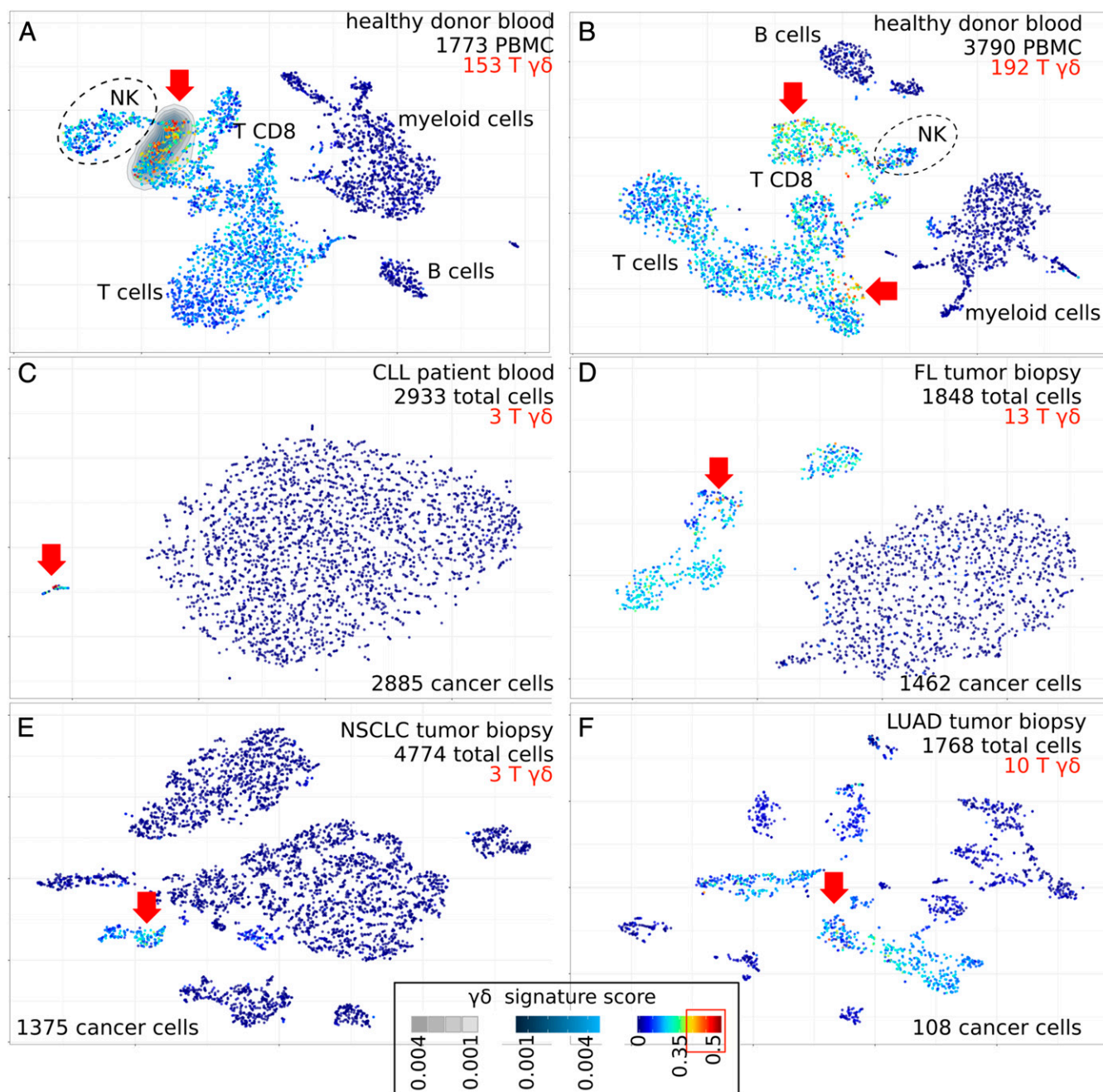
Fig. 3. Identifying γδ T lymphocytes in t-SNE plots of other PBMC and tumor samples. (*A* and *B*) Same visualization as above for γδ T lymphocytes (orange-red dots) from other examples of PBMC from two healthy donors, (*C*) blood cells from a relapsed CLL patient (present study), (*D*) tumor biopsy from a diagnosed FL patient (36), (*E*) and tumor biopsies from a patient with NSCLC, and (*F*) from a patient with LUAD (66). Arrows: γδ T cells. The cell clusters of PBMC are specified.

620 TILs (1.6% of TIL) were detected in a lung adenocarcinoma (LUAD) biopsy (Fig. 3 *E* and *F* and *SI Appendix*, Table S2).

Distinct Clusters for TCRVδ1 and TCRVδ2 γδ T Lymphocytes. The distinct partitions of *TRGC1* and *TRGC2* gene expression in the 12k PBMC t-SNE plot (*SI Appendix*, Fig. S1*A*) suggested that blood γδ T lymphocytes expressing TCRVγ9Vδ2 (encoded by *TRGC1* and *TRDC*) and those expressing TCRVγ(non-9)Vδ1 (encoded by *TRGC2* and *TRDC*) have different transcriptomes. To validate this observation, the above 1,333 purified γδ T lymphocytes were analyzed separately according to their cell surface TCRVδ1 or TCRVδ2. These encompassed $n = 570$ of

641 purified TCRVδ1 γδ T lymphocytes with a mean of 155k reads for 1,374 unique genes per cell, and $n = 763$ of 888 purified TCRVδ2 γδ T lymphocytes, with a mean of 108k reads for 1,323 unique genes per cell. In the high-resolution t-SNE plot of the integrated 13k dataset, these cells segregated in neighboring but distinct regions: the TCRVδ2 T lymphocytes mapped next to the mature CD8 T cells, whereas the TCRVδ1 T lymphocytes were closer to the NK cells. Unsupervised hierarchical clustering of the 13k dataset confirmed this partition (*SI Appendix*, Fig. S3). Of note, the expression levels of *TRGC1* and *TRGC2* was inversed between the two subsets of purified γδ T cells [*TRGC1* mean (log$_2$ unique molecular identifier [UMI] = 0.9 in

TCRVδ1⁺ cells versus 1.7 in TCRVδ2⁺ cells, *TRGC2* mean = 2.3 in TCRVδ1⁺ cells versus 0.8 in TCRVδ2⁺ cells)]. Thus, their significantly different (*TRGC1-TRGC2*) expression values (Student $P = 10^{-235}$) characterized the TCRVδ1 and TCRVδ2 subtypes of γδ T cells identified in the above-depicted datasets (*SI Appendix*, Table S2). Hence, the 515 γδ T cells from the 12k PBMC comprised $n = 195$ TCRVδ1, $n = 244$ TCRVδ2, and $n = 76$ TCR-unassigned γδ T lymphocytes. From this and three additional datasets totaling 28,339 PBMC (*SI Appendix*, Table S2), there were a total of $n = 263$ TCRVδ1 cells (on average 0.9% of PBMC), $n = 460$ TCRVδ2 (on average 1.6% of PBMC), and $n = 197$ TCR-unassigned γδ T lymphocytes (~0.6% of PBMC), totaling $n = 920$ γδ T lymphocytes or 3.2% of PBMC as expected in normal blood. However, in 26 cancer samples totaling 25,658 cells, the γδ TIL were consistently more scarce, $n = 115$ γδ T lymphocytes (0.4% of total cells), and were mostly of TCRVδ1⁺ subtype ($n = 52$ lymphocytes, 0.9% of total cells; $n = 8$ TCRVδ2 cells; $n = 55$ TCR-unassigned γδ T). In addition, although γδ TIL counts and subsets varied between patients, the γδ T lymphocytes detected in lung cancer samples often came from their normal adjacent tissue. Finally, across these cancer samples, the rates of γδ TIL and αβ TILs were not correlated (Pearson $r = 0.12$) (*SI Appendix*, Table S2), as reported previously (28).

Shared and Distinct Cytotoxic Genes of TCRVδ1 and TCRVδ2 γδ T Lymphocytes. Together, the $n = 1,265$ purified TCRVδ1 and TCRVδ2 γδ T cells mapped in the γδ cell area of the t-SNE had

470 differentially expressed genes compared with the ($n = 7,071$) αβ T lymphocytes (both CD4 and CD8 T cell clusters from the 13k integrated dataset), with significantly higher expression of the cell cytotoxicity genes. They also had 570 differentially expressed genes relative to the ($n = 493$) NK cells from the NK cluster, with significantly stronger expression of the T cell-specifying genes (*SI Appendix*, Tables S3 and S4), consistent with earlier microarray studies (29).

At the single-cell level, few genes were selectively expressed by most γδ T cells relative to all of the rest of the PBMC. These included *FGFBP2* encoding for the cytotoxic serum protein fibroblast growth factor-binding protein 2, *MT2A* encoding for metallothionein 2A, *CST7* encoding for the cysteine protease inhibitor Cystatin F, *HOPX* encoding for the homeobox transcription repressor X, *GZMM* encoding for granzyme M, and *KLRD1* encoding for the cell cytotoxicity coreceptor CD94, although these genes were also expressed by some cells from other subsets of PBMC (*SI Appendix*, Fig. S4). Typically, 99% of the purified γδ T lymphocytes from donor #1, but also 99% of NK cells and 93% of mature cytotoxic (*FCGR3A⁺*) CD8 T lymphocytes from the 13k dataset expressed *NKG7* encoding for the NK cell granule protein 7 (Fig. 4*A*). Similar results were observed in additional PBMC datasets (*SI Appendix*, Table S2). Most purified γδ T lymphocytes also expressed mRNA encoding for several others typical cytotoxic but not γδ T cell-specific mediators, such as *GZMM* (87% of cells), the granulysin-encoding *GNLY* (78% of cells), and the perforin-encoding *PRF1* (66% of cells),
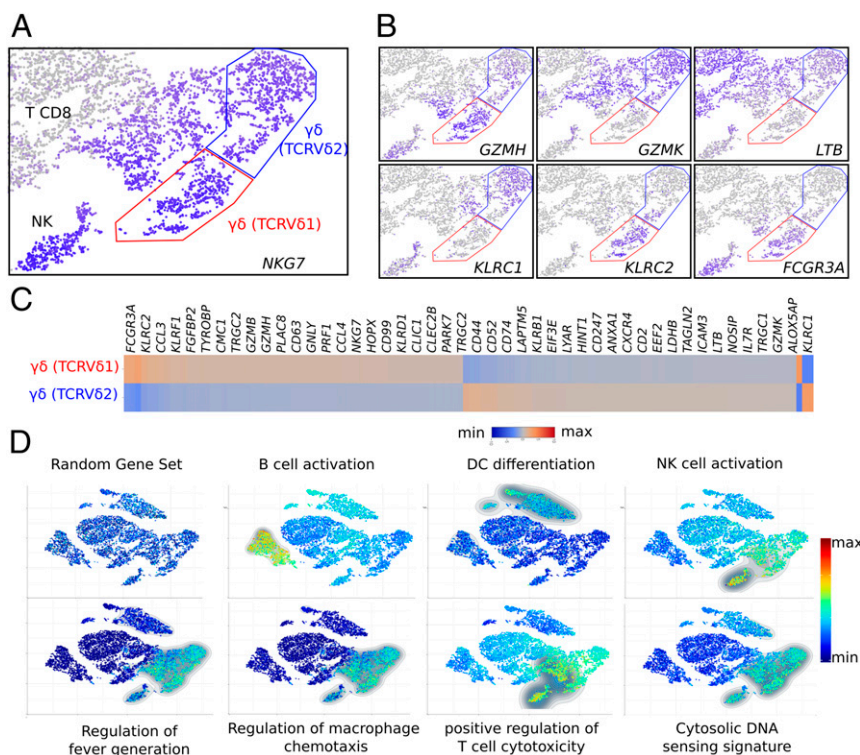
Fig. 4. Distinctive gene-expression patterns of TCRVδ1 and TCRVδ2 γδ T lymphocytes from CMV⁺ donor #1. (*A*) Zoom-in of the same cytotoxic cell area of t-SNE plot as in Fig. 1, shown with purple color-encoded expression level of *NKG7* gene across the NK cells, CD8 T lymphocytes, TCRVδ1, and TCRVδ2 γδ T lymphocytes. (*B*) Same as in *A* for expression of the specified genes. (*C*) Representative genes differentially expressed between TCRVδ1 and TCRVδ2 γδ T lymphocytes. (*D*) Functional enrichment of the specified signatures in each single cell from the integrated PBMC and purified γδ T cells dataset is shown by featuring the single-cell enrichment score (i.e., the proportion of UMI for genes from the signature and expressed by each cell divided by the total UMI of the cell) (*SI Appendix, Material and Methods*), and contour plots of the region with differentially enriched signature over the t-SNE plot (region of cells having signature score >2× mean score of t-SNE). They comprise a negative control signature (12 randomly selected genes, RGS407_12 from this study), B cell activation [132 genes, gene ontology (GO)], dendritic cell differentiation (33 genes, GO), NK cell activation (55 genes, GO), regulation of fever generation (11 genes, GO), regulation of macrophage chemotaxis (16 genes, GO), positive regulation of T cell cytotoxicity (16 genes, GO), cytosolic DNA sensing: (56 genes, Kyoto Encyclopedia of Genes and Genomes). The corresponding genes are listed in *SI Appendix, Table S6*.

to quote a few. Similar expression rates (*GZMM*: 80%, *GNLY*: 91%, *PRF1*: 86%), were found for the bulk γδ T lymphocytes from the 12k PBMC scRNA-seq dataset. Consistent with the TCRVδ2 T lymphocyte maturation pathway depicted previously (13, 14), both FACS- purified TCRVδ1 and TCRVδ2 cells expressed variable levels of the maturation marker genes *ITGAL* (CD11b) and *SELL* (CD62L) characterizing naïve T cells, as well as *CD27*, *CD28*, and *CD44* shared by central and effector memory T cells, and *FCGR3A* (encoding for CD16) typifying the TEMRA T lymphocytes (Fig. 4*B* and *SI Appendix*, Figs. S4 and S5).

Overall, 38 genes were differentially expressed between the (*n* = 570) purified TCRVδ1 and (*n* = 763) purified TCRVδ2 γδ T lymphocytes from CMV⁺ donor #1. These comprised 25 genes, such as *KLRC2* (NKG2A), *KLRF1* (NKp80), *FCGR3A* (CD16), *GZMB*, and *GZMH*, which were up-regulated in TCRVδ1 cells, and 13 genes, including *KLRC1* (NKG2C), *GZMK*, *LTB*, and *IL7R*, which were up-regulated in TCRVδ2 cells (Fig. 4 *B* and *C* and *SI Appendix*, Table S5). These gene-expression profiles were consistent with the immunostaining and flow cytometry phenotypes of the γδ T lymphocytes from the same CMV⁺ PBMC donor (*SI Appendix*, Fig. S5). Nevertheless, the clear-cut segregation of *KLRC2*/*KLRC1* expression by the TCRVδ subsets from this CMV⁺ PBMC donor #1 could not be generalized, as it disappeared when examining the single γδ T cell transcriptomes from additional scRNA-seq datasets (*SI Appendix*, Table S2) and when phenotyping the γδ PBMC from 15 additional CMV⁺ kidney transplant recipients (*SI Appendix*, Fig. S6).

The gene-expression programs of all cells from the 13k PBMC dataset were finally visualized by scoring each single cell for expression of functionally defined pathways, and comparing these single-cell scores over the t-SNE. In control tests, no subset of PBMC showed up-regulation of random gene signatures, while the B cell activation signature was found in B cell clusters, and the DC differentiation signature was found in myeloid cell clusters, as expected. This analysis revealed that among the most salient signatures of the γδ T lymphocytes, and to some extent of the surrounding NK and CD8 T cell clusters, were "NK cell cytotoxicity," "positive regulation of T cell cytotoxicity," "regulation of fever generation," "regulation of macrophage chemotaxis," and "cytosolic DNA sensing" signatures (Fig. 4*D* and *SI Appendix*, Table S6). In contrast, no curated signature was found specific for either of the purified TCRVδ1 or the purified TCRVδ2 γδ T cell subsets in these analyses. Thus, these two subsets of γδ T lymphocytes share a cytotoxic signature comprising both common and distinct genes.

Similar Pseudotemporal Maturation Trajectories of TCRVδ1 and TCRVδ2 γδ T Lymphocytes Unveil Mature γδ T Cells with Proliferative Capacity. The fate of peripheral γδ T lymphocytes relies on a maturation process driven by antigen and cytokine stimulation. This maturation starts from naïve resting cells that evolve to central memory cells able to proliferate, and may further mature into effector memory cells, which produce either IFN-γ or Perforin, and finally terminally differentiated, CD45RA⁺CD16⁺ cytotoxic cells with short telomeres, named TEMRA (13–15). Whether this seemingly linear maturation trajectory is validated by an unsupervised algorithmic derivation of high-resolution differentiation trajectories deserved clarification. Furthermore, whether Vδ1 and Vδ2 γδ T cells follow the same scheme is unclear (22, 38). To address these issues, the purified γδ T cells of the 13k PBMC dataset and the bulk cells colocalized within the purified γδ T lymphocyte clusters from t-SNE plot (*n* = 1,683 lymphocytes) were selected and their TCRVδ lineage was inferred on the basis of *TRGC1* and *TRGC2* expressions. A pseudotemporal trajectory of maturation was then reconstructed using the FateID package (39), which orders topologically the successive expression profiles, and draws a differentiation trajectory fitting to all ordered cells, visualized by a self-organizing pseudotemporal map. This revealed that TCRVδ1 and

TCRVδ2 γδ T lymphocytes display quite parallel maturation trajectories (Fig. 5*A*). Both subsets expressed the maturation-defining genes *CCR7*, *IL7R*, *SELL*, *ITGAL*, and *CD27* at the earliest stages only, while the cytotoxicity genes *NKG7*, *PRF1*, *NCR3*, *GZMM*, and *FGFBP2* increased at later time points. Both subsets also encompassed *FCGR3A*-expressing mature cells, although more frequently among the TCRVδ1 than the TCRVδ2 γδ T lymphocytes from CMV⁺ donor #1, suggesting their more advanced cytotoxic maturation (Fig. 5*B*).

Chronological ordering of the *z*-score–transformed pseudotemporal expression profiles of the TCRVδ1 and TCRVδ2 γδ T lymphocytes identified several nodes of gene coexpression (Fig. 5*C*). For each cell subset, the gene signatures significantly enriched at all these nodes were identified as above. This analysis revealed two mitotic windows at early, presumably naïve/central memory stages, and at late mature/TEMRA stage (Fig. 5*C*). Consistent with this finding, flow cytometry analysis of far red cell tracker (CFTR) dilution by PBMC stimulated in vitro with either PHA/IL-2 as TCRVδ1-stimulus (22) or BrHPP/IL-2 as TCRVδ2-stimulus (40) confirmed that some TEMRA γδ T lymphocytes had proliferated after 8 d of in vitro culture. Although in each subset the divided cells were mostly naïve and central memory lymphocytes, as expected from earlier literature (13–15), both PHA/IL-2–stimulated TCRVδ1 and BrHPP/IL-2–stimulated TCRVδ2 γδ T lymphocytes from healthy donors comprised some terminally differentiated γδ lymphocytes with mitotic capacity, as evidenced by the diluted intracellular CFTR of such cells with intermediate CD45RA phenotype after 14 d of culture (*SI Appendix*, Fig. S7). To confirm that TEMRA cells are able to proliferate, CMV-infected kidney graft recipient's PBMC with TCRVδ1 γδ T cells composed of more than 90% of TEMRA cells were cultured with IL-2 and IL-15. After 7 d of culture, the number of TCRVδ1 T cells was increased by 10-fold (*SI Appendix*, Fig. S8*A*) and these were still predominantly TEMRA cells (*SI Appendix*, Fig. S8*B*). CFSE [5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester] staining of PBMC and gating of TEMRA cells only at day 7 confirmed that TEMRA TCRVδ1 cells were able to proliferate in the presence of IL-2 and IL-15 but did not proliferate in the absence of activation (*SI Appendix*, Fig. S8*C*).

Hence, the pseudotemporal maturation trajectories correctly identified the terminally differentiated TCRVδ1 and TCRVδ2 γδ T lymphocytes, which maintained a proliferative capacity.

Maturation Stages of TCRVδ1 and TCRVδ2 γδ T Lymphocytes from CMV⁺ and CMV⁻ Donors. The above results from a single γδ donor of CMV⁺ status questioned their generalization. To address this, the circulating TCRVδ1 and TCRVδ2 cells were purified from two additional donors of known CMV status, and their scRNA-seq were performed and analyzed as above. For the CMV⁺ donor #2, these encompassed *n* = 1,775 of 1,800 purified TCRVδ1 γδ T lymphocytes totalizing 13,241 unique genes and a median of 1,311 unique genes per cell, as well as *n* = 1,277 of 1,306 purified TCRVδ2 γδ T lymphocytes, totaling 12,787 unique genes and a median of 1,370 unique genes per cell. For the CMV⁻ donor #3, these encompassed *n* = 1,803 of 1,825 purified TCRVδ1 γδ T lymphocytes totaling 13,335 unique genes and a median of 1,197 unique genes per cell, as well as *n* = 1,720 of 1,750 purified TCRVδ2 γδ T lymphocytes totaling 13,283 unique genes and a median of 1,419 unique genes per cell. Altogether, these (*n* = 6,575) additional purified γδ T lymphocyte datasets were integrated, aligned, and processed with the previous 13k dataset, yielding a 20k dataset that was visualized by high-resolution t-SNE plot, as above. Scoring and projecting the γδ signature across the 20k t-SNE, which included 7,150 γδ T cells, both 6,575 purified and the 575 γδ T already in the 12k PBMC (*SI Appendix*, Table S7) identified a total of *n* = 5,995 γδ T lymphocytes (84% sensitivity). As previously, these cells neighbored both CD8 T and NK cells and each donor yielded two

Fig. 5. Pseudotemporal reconstruction of the γδ T cell maturation trajectory. (*A*) Tridimensional t-SNE representation of γδ T cell data after classic multidimensional scaling and dimensionality reduction. Data shown represent *n* = 1,683 cells, and principal curves fitted to either the TCRVδ1 γδ T lymphocytes (red line) or the TCRVδ2 γδ T lymphocytes (black line). Cells are colored according to trajectory subclusters. (*B*) Gene-to-gene scatterplots of marker genes expression in the TCRVδ1 γδ T lymphocytes and TCRVδ2 γδ T lymphocytes from CMV⁺ donor #1 across their respective maturation trajectories. The average level is shown in bold line. (*C*) Self-organizing map of z-score–transformed pseudotemporal coexpression nodes profiled along the respective maturation trajectories of TCRVδ1 γδ T lymphocytes and TCRVδ2 γδ T lymphocytes. The areas (dotted rectangle) labeled "m" show maturation stages with coexpression nodes (arrows) significantly enriched for mitosis/cell cycle signatures. The bottom color bar shows the appearance order of each maturation subcluster of cells, using the same color keys as in *A*.

distinct clusters of γδ T lymphocytes corresponding to each TCRV subset (*SI Appendix*, Fig. S9*A*).

In this t-SNE, the cytotoxic hallmark genes *NKG7*, *GZMH*, *GZMK*, *KLRC1*, and *FCGR3A* were expressed by the γδ T lymphocytes from all donors, except *KLRC2*, which was only expressed by the *FCGR3A* (TEMRA) TCRVδ1 cells from donor #1 (*SI Appendix*, Fig. S9*B*). For donors #2 and #3, the genes differentially expressed between TCRVδ1 and TCRVδ2 γδ T lymphocytes (*SI Appendix*, Tables S8 and S9) were reminiscent of those reported above for donor #1, and reflected their respective maturation stage in each donor. In t-SNE of purified γδ T cells alone, the embedding of TCRVδ1 and TCRVδ2 γδ T cells from the two CMV⁺ donors #1 and #2 indicated these subsets share transcriptional hallmarks that TCRVδ1 and TCRVδ2 γδ T cells from the CMV⁻ donors #3 do not (*SI Appendix*, Fig. S9*C*). The respective cell counts of the naïve, central memory, effector memory, and effector memory RA cells in each TCRVδ subset from each donor accounted for the above results (*SI Appendix*, Table S10). These data unveiled the prominence of mature TCRVδ1 cells in CMV⁺ donor #1 and of mature TCRVδ2 cells in CMV⁻ donor #3, while CMV⁺ donor #2 shared both hallmarks with each respective donor (*SI Appendix*, Fig. S9*D*).

Thus, in healthy individuals, the single-cell transcriptomes of circulating TCRVδ1 and TCRVδ2 γδ T lymphocytes merely reflect their immune and CMV-driven maturation.

Discussion

The identification of human γδ T lymphocytes is a major issue for cancer immunotherapy, as these pleiomorphic cells infiltrating most healthy organs are often misidentified when not unidentified at all. In this aim, detection of γδ T lymphocytes classically relies on a combination of cell surface markers labeled by immunostaining and visualized by flow cytometry or immunohistochemistry. These techniques require access to fresh tumor samples and, furthermore, immunohistochemical spotting of cells expressing the γδ TCR remains largely hampered by the paucity of anti-TCR reagents compatible with staining of formalin-fixed paraffin-embedded samples. However, the recent developments in scRNA sequencing are updating these methodologies, and they provide us with the finest level of resolution for the gene-expression patterns of large sets of cells, unveiling much finer aspects of cells and allowing their classifications from human tissues.

We report here scRNA-seq datasets of human γδ T lymphocytes as bulk cells among unsorted PBMC, and as highly purified lymphocytes. These transcriptomes were integrated to those of a pool of several bulk PBMC, because t-SNE plots from larger datasets provide a wider and more comprehensive view of all cell components from complex tissues. In addition, t-SNE hyperparameters, such as clusterization level, determine the visualization of the underlying data output by this nondeterminist algorithm. These two features raise the issue of spotting in large t-SNE maps those rare subsets of cells, like γδ T lymphocytes, which transcriptomes resemble those of different and more abundant cell types, and without any selectively identifying gene. This issue was exemplified here by the strikingly high *TRDC* gene expression by NK cells, a hitherto unreported (34) transcriptional hallmark supporting the innateness gradient of lymphoid cells (41). In the absence of techniques such as the recently developed CITE-Seq combining scRNA-seq and detection of cells displaying surface markers (33), which fails at detecting the γδ TCR, spotting the γδ T lymphocytes was elusive, and actually

never done in published scRNA-seq studies. This pitfall is typically exemplified by recently improved scRNA-seq processing algorithms, which outperform the other softwares for execution time, clustering accuracy, and detectability of minor cell subtypes (*SI Appendix, Material and Methods*), but still fail at spotting any γδ T lymphocytes among ~68,000 PBMC scRNA-seq (42). We show here that identification of γδ T lymphocytes requires not only large datasets and highly granular t-SNE plots, but also a γδ signature combining γδ T-scoring with NK and T CD8-discarding genes. This allowed us to detect the γδ T lymphocytes and identify their subsets in healthy individuals PBMC and in tumor biopsies from cancer patients. Our study represents a resource to identify γδ T lymphocytes from human tissues, as undertaken by the human cell atlas project (1), and to determine their fonctional status in cancer patients under immunotherapies.

The second conclusion of this study is the signature of γδ T from PBMC that still remains at the single-cell level a profound blend in of the cytotoxic signatures of mature CD8 T and NK cells. Collectively, this cytotoxic hallmark is today well documented through microarrays and phenotypic studies of bulk TCRVδ1 T lymphocytes and TCRVδ2 T lymphocytes, taken separately (29, 43–49). As a consequence, several clinical trials in cancer are currently aimed at assessing the therapeutic activity of allogeneic or autologous TCRVδ1 or TCRVδ2 γδ T cell transplants produced by various methodologies. Nevertheless, these earlier studies had demonstrated that TCRVδ1 T lymphocytes and TCRVδ2 T lymphocytes mediate distinct cytotoxic responses. In particular, cytotoxicity-controlling receptors, such as NKG2A, NKG2C, NKG2D, NKp30, and CD85, were reported as a differential hallmark of these two main subsets of γδ T cells (22, 24, 50). Indeed, although a segregation of *KLRC1*, *2*, and *GZMB*, *H* expressions according to the TCRVδ subset can be occasionally seen as in CMV^+ donor #1, this differential hallmark was not a general rule. Instead, the NK receptor pattern displayed by the various γδ T cell subsets could reflect distinct maturation stages driven by prior exposure to activating ligands (reviewed in ref. 24), as shown by the gene-expression profiles along maturation trajectories. Pioneering studies (48, 51) have demonstrated age-related changes in the γδ repertoire. TCRVδ2 γδ T cells are a minority of blood γδ T cells at birth, but expand later to constitute the majority of γδ T cells in adults blood while TCRVδ1 γδ T cells have no such expansion. Recent RNA-seq studies (19, 20) have shown that while the TCRVδ2 repertoire remains almost equivalent in cord and adult blood, the private and unfocused TCRVδ1 repertoire in cord blood strongly focuses in few high-frequency adult clonotypes. Such clonal expansions are accompanied by maturation from naive to effector phenotypes, consistent with the gene-expression profiles promoted by physiological immune processes (52), exemplified here by the different TCRVδ1 transcriptomes of the CMV^+ and CMV^- donors.

Beyond cytotoxic hallmarks, however, our study also detected enrichment of a gene signature dedicated to regulation of fever generation in the single cells of γδ T lymphocytes. This finding was unattended for human blood γδ T cells, but fully consistent with the recent discovery that mice lacking γδ T lymphocytes cannot regulate their core body temperature (53). Taken together, those observations raise the emerging possibility that, like their invariant NK T cell counterparts, the γδ T cells might fulfill physiological functions of immune-mediated regulation of body metabolism (54).

Despite the current model stating that terminally differentiated γδ T lymphocytes have progressively lost their proliferative capacity (13–15), the third conclusion of this study is that some terminally differentiated TEMRA cells from both TCRVδ1 and TCRVδ2 subsets of γδ T lymphocytes have kept a significant proliferative capacity. This proliferative capacity remains in a CD45RA^{intermediate} subpopulation of TEMRA cells, however, fully consistent with a recent report depicting two populations of TEMRA TCRVδ2 cells in solid cancer patients (55). This unattended finding arose from the unbiased algorithmic spotting of mitotic gene signatures in cells positioned at the end of chronologically ordered maturation trajectories, and was validated by further flow cytometric analyses and in vitro proliferation assays. On the one hand, such results illustrate the resolutive power of the scRNA-seq technologies that were not available when the first γδ maturation model was elaborated, and on the other hand they prompt new maturation models. These findings also reconciles the previously unexplained—although largely used—capacity to grow in vitro large-scale and clinical grade batches of terminally differentiated TCRVδ2 γδ T lymphocytes for cell-therapy purposes in patients with metastatic renal cell carcinoma (56), of TCRVδ1 γδ T lymphocytes for cell therapy of chronic lymphocytic leukemia (25), or total γδ T cells for cell therapy in neuroblastoma (57) or in ovarian cancer (58). Beyond cancer, mature γδ T lymphocytes contribute to control infections such as HIV disease (59) and CMV reactivation (60, 61). Therefore, terminally differentiated TCRVδ1 and TCRVδ2 γδ T lymphocytes can improve the prevention of early relapse after allogeneic hematopoietic stem cell transplantation (62), and are developed for cell therapy by haploidentical mature γδ T cell infusions (63). Newer developments of expanding cell batches of terminally differentiated γδ T lymphocytes are also emerging for CAR-T cell therapy (64), and will benefit of the present in-depth characterization of these γδ T lymphocytes at the single cell level (65).

Materials and Methods

Informed consent and ethical approval was obtained for blood samples (Bordeaux University Hospital; ethical committee agreement 2013/57). The isolation and phenotyping of PBMC and purified γδ T lymphocytes, in vitro proliferation assays, scRNA sequencing and analyses, including single-cell signatures and scores, maturation trajectories, additional datasets, and statistical analyses are described in *SI Appendix, Material and Methods*.

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mTOR inhibitors prevent CMV infection through restoration of functional αβ and γδ T cells in kidney transplant recipients

One sentence summary: Reduced CMV incidence and severity by mTORi in kidney transplant recipients is associated with reinvigorated αβ and γδ T cell profile.

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Abstract

Association of mTOR-inhibitors (mTORi) treatment with a lower incidence of cytomegalovirus (CMV) infection in CMV-seropositive (R+) kidney transplant recipients (KTR) remains unexplained. We hypothesized that CMV infection in R+ patients could be due to dysfunctional T cells that might be improved by mTORi. First, in R+ KTR receiving mycophenolic acid (MPA), we showed that both αβ and γδ T-cells displayed a more dysfunctional phenotype (LAG3+, TIM3+, PD-1+, CD85j+) at day 0 of transplantation in the 16 KTR with severe CMV infection when compared to the 17 KTR without or with spontaneously resolving CMV infection. Second, in patients treated with mTORi (n= 27), the proportion of PD-1+ and CD85j+ αβ and γδ T-cells decreased when compared to MPA-treated patients (n=44), as the frequency and severity of CMV infections. mTORi treatment also led to higher proportions of late-differentiated and cytotoxic γδ T-cells, IFNγ producing and cytotoxic αβ T-cells. *In vitro*, mTORi (i) increased proliferation, viability and CMV-induced IFNγ production of γδ and αβ T-cells, (ii) decreased PD-1 and CD85j expression in both subsets that shifted to a more efficient EOMES^{low} Hobit^{high} profile. In γδ T-cells mTORi effect was related to increased TCR signaling. Our results reveal (i) that severe CMV replication is associated with a dysfunctional T cell profile and (ii) that mTORi improve their fitness in association with a better control of CMV. Dysfunctional T cell phenotype could represent a new biomarker to predict post-transplantation infection if KTR received MPA and to stratify patients who should benefit from mTORi treatment.

Introduction

CMV is by far the most common opportunistic infection in solid allograft recipients and induces direct and indirect morbidity (1). The risk to develop post-transplant CMV infection mainly relies on graft donor and recipient (D/R) CMV serostatus, and seropositive donor/seronegative recipient (D+R-) confers the highest risk. CMV seropositivity of the recipient (R+) is associated with an intermediate risk of CMV reactivation or superinfection. It concerns the vast majority (50-90 %) of transplant recipients over the world (2). A preformed cell-mediated immunity (3, 4) contributes to this reduced risk, but sometimes fails to control the virus, leading to clinical infections. A dysfunctional status of CMV-specific T cells is one hypothesis to explain the emergence of CMV infection and disease in some CMV seropositive patients after transplantation.

CD8+ $\alpha\beta$ T cell response during CMV infection was described as inflationary both in mice models and in humans, since it leads to the lifelong accumulation of highly differentiated T cells (5, 6) that remain functional and correlate to chronic but well-controlled low-level viral load (7). The specific phenotype of these CMV-specific T cells is characterized by a highly differentiated status, strong activation marker and NK cell receptor expression (5). Moreover, among these highly differentiated T cells, a "long-lived effector-type" phenotype has been distinguished for cells expressing the transcription factor Hobit and having strong capacities for both IFNγ and granzyme B production and for self-renewal (8-10).

Conversely, a dysfunctional profile of effector T cells has been deeply characterized in chronic lymphocytic choriomeningitis virus (LCMV) infection with clone C13 in mice (and also in humans during chronic hepatitis C, B and HIV) in which high virus load persisted and was involved in the progressive hypo-responsiveness of antigen-specific effector T cells (for review see (5)). Dysfunctional state is characterized by an increased expression of inhibitory receptors

79   such as PD-1, a transcriptional program balance with high level of EOMES/low level of T-bet

80   and by a decreased ability to produce cytokines and to proliferate (11). Conversely, "T-

81   bet[high]/EOMES[low]/PD-1^{low}" effector T cells are more functional (11, 12).

82   Our hypothesis is that multiple CMV reactivations or higher viral loads could occur in

83   immunocompromised patients when compared to immunocompetent individuals and could lead

84   to a progressive hypo-responsiveness of specific T cells, favoring the emergence of such

85   dysfunctional CMV-specific T cells (9, 13-18). In kidney transplant recipients (KTR), such

86   hypo-responsiveness could be related to end-stage renal disease (19) and/or to the

87   immunosuppressive drugs required to prevent allograft rejection. Interestingly, among the

88   different immunosuppressive treatments that can be used in organ transplantation, mTOR

89   inhibitors (mTORi) have been recently associated to a decreased number of CMV events (20-

90   22). mTORi are now common components of immunosuppressive regimen, due to their

91   antiproliferative effects on immune cells (23), including alloreactive naive T cells (24).

92   The association of mTORi with reduced risk of CMV infections remains unexplained. It could

93   be due to reportedly direct antiviral action but this effect has only been observed *in vitro* and

94   not in all type of CMV-infected cells (25). Alternatively, it could be due to an effect on CMV-

95   specific immunity. Indeed, whereas mTORi have a strong anti-proliferative effect on naive cells

96   (24), they also increase CD8 memory T cells maintenance after LCMV infection in mice (26).

97   However, data on mTORi effect on human T cells remains elusive particularly on highly

98   differentiated CMV-specific T cells.

99   Since mTORi positive association with reduced CMV events has been observed in R+ patients

100 (21) but not in D+R- patients (27), we hypothesized that mTORi could improve the functions

101 of preformed CMV-specific effector T cells.

102   T cell response against CMV involves both CD8+ $\alpha\beta$ and $\gamma\delta$ T cells (28). In humans, $\gamma\delta$ T cells

103   are classically divided according to the TCR chains they express: Vγ9Vδ2 TCR-expressing

104   cells are opposed to the others, collectively called Vδ2^{neg} $\gamma\delta$ T cells (expressing mainly Vδ1

105   but also Vδ3 to Vδ8 chains). Only Vδ2^{neg} $\gamma\delta$ T cells are involved in the control of CMV, as

106   demonstrated in many different studies (for review(29)), and their response is very similar to

107   that of CMV-specific CD8+ $\alpha\beta$ T cells. They share similar kinetics, acquire the same late

108   effector memory CD45RA+ (TEMRA) phenotype and antiviral functions (cytotoxicity, IFNγ

109   production) (30, 31). However, in contrast with $\alpha\beta$ T cells, Vδ2^{neg} $\gamma\delta$ T cells do not recognize

110   viral peptides presented by HLA molecules, but CMV-induced "stress-self-antigens" at the

111   surface of infected cells. Very few of these antigens have been described (32).

112   In the present study, our first aim was to analyze the phenotype of CMV-specific $\alpha\beta$ and $\gamma\delta$ T

113   cells in CMV-seropositive kidney recipients to assess if specific attributes before

114   transplantation could be associated with the risk of developing CMV reactivation after

115   transplantation. Then, we investigated *in vitro* and *in vivo* the effect of mTORi on the function

116   and phenotype of CMV-specific effector $\alpha\beta$ and $\gamma\delta$ T cells in order to understand why this

117   immunosuppressive drug is associated with a better control of CMV infection in patients.

118

Results

Increased percentages of γδ T cells and CMV-specific CD8+ T cells expressing inhibitory receptors at baseline in patients with severe CMV infections.

We first explored the hypothesis that preformed T cells in CMV seropositive (R+) patients could present a dysfunctional profile. To this end, we characterized γδ T cells and total CD8+ αβ T cell phenotype at day 0 of transplantation (baseline phenotype) in a cohort of 21 KTR treated with MPA. Late-differentiated CD45RA+ T effector cells (TEMRA) were highly represented in γδ T cells and CD8+ T cells (**Figure 1A**) as previously observed (31). Both T cell compartments presented high percentages of cells expressing activation receptors such as DNAM-1 (CD226) (also CD8αα and CD16 for γδ T cells), as well as KLRG1, a receptor expressed on highly differentiated CD8+ T cells with high cytotoxic but low proliferative capacities in CMV infection (33). Interestingly, a significant proportion of T cells also expressed inhibitory receptors, such as CD85j, PD-1 and TIM3 (also CD161 and LAG3 for γδ T cells) (**Figure 1B**). These phenotypes were then compared between patients with post-transplant CMV DNAemia requiring an antiviral treatment (considered as *severe CMV infections* in the rest of the paper) (n=9) and patients with *well-controlled CMV*, *i.e.* those with either no post-transplant CMV DNAemias or with spontaneous CMV DNAemia negativation without any antiviral treatment (n=12). No difference in the differentiation status groups (**Supplemental Figure 1A**) or in expression of KLRG1, DNAM-1, CD8αα and CD16 (**Figure 1C**) were observed between each group. However, patients who will present a severe CMV infection had a higher percentage of T cells expressing inhibitory receptors, such as PD-1 and CD85j when compared to patients with well-controlled CMV DNAemias (**Figure 1C**). Finally, we validated and extended these results by analyzing concomitantly γδ T cells, total CD8+ T cells (**Supplemental Figure 1B**) but also CMV-specific CD8+ T cells in an internal validation

144   cohort of 12 additional patients. We thus confirmed that patients with severe CMV infection

145   also displayed significantly higher percentages of PD-1+, and CD85j+ cells in CMV-specific

146   CD8+ T cells (**Figure 1D)** and validated those markers in total CD8+ and γδ T cells

147   (**Supplemental Figure 1B**).

148   Thus, at baseline, γδ , total CD8+ and CMV-specific CD8+ T cells from CMV seropositive

149   patients could display a pre-existing dysfunctional profile, characterized by expression of

150   inhibitory receptors, which correlate to severe CMV infection after transplantation.

151

152   **The proportion of functional T cells is enhanced by mTORi treatment and correlates to a**

153   **subsequent lower incidence of CMV infection**

154   To address the issue of mTORi effect on dysfunctional T cells, 44 KTR patients treated with

155   mycophenolic acid (MPA) were compared to 27 KTR patients treated with mTORi

156   (everolimus). First, we confirmed that mTORi treatment protects from CMV-infection since 26

157   % (7/27) of patients treated with mTORi displayed CMV infection during the first year of

158   transplantation, sharply contrasting with 70 % (32/44) in patients treated with MPA (p<0.001)

159   **(Figure 2A**).

160   At day 14 post-transplantation (prior to any CMV replication), total and TEMRA γδ T cell

161   percentages were higher in mTORi-treated patients than in MPA-treated patients (p=0.04 and

162   p=0.005, respectively) (**Figure 2B and 2C**). At day 14 also, CMV-specific αβ T cell immunity

163   assessed by QuantiFERON-CMV was also higher in mTORi-treated patients compared to

164   MPA-treated patients (**Figure 2D**). Then, we compared the effect of MPA (n=8 patients) and

165   mTORi (n=7 patients) on the evolution of T cell phenotype before any CMV replication.

166   mTORi treatment was associated with a decreased percentage of PD-1+ and CD85j+ cells

167   (except in γδ T cells for CD85j where it was unchanged) while MPA was associated to increased

168 or stable PD-1+ and CD85j+ cell percentages (**Figure 2E**). mTORi treatment was also

169 associated with a decrease of KLRG1+ cells among γδ T cells, and an increase of perforin+

170 cells in all T cell subsets (**Figure 2E**). Importantly, there was a direct association between the

171 decrease of CD85j- or PD1-expressing T cells percentage during the first month of

172 transplantation and an absence or a low (<1000 IU/ml) CMV DNAemia (independently of MPA

173 or mTORi treatment) (**Figure 2F**).

174 To sum up, mTORi treatment increases the percentages of γδ T cells and CMV-specific αβ T

175 cells expressing a functional profile which is associated to a low incidence of CMV infection

176 post-transplantation.

177

178 **mTORi treatment is associated with milder CMV infections that correlate to better T cell**

179 **response to the infection than MPA.**

180 We then focused on the patients who developed post-transplantation CMV infection. Viral load

181 was much lower (never exceeding 999 IU/ml) after treatment with mTORi (n=7; mean 999

182 IU/ml; standard deviation 0) than with MPA (n=32; p=0.015; mean 7482 IU/ml; standard error

183 11887 IU/ml) (**Figure 3A**). Moreover, mTORi-treated patients did not require any anti-viral

184 treatment since they all spontaneously cleared the virus by contrast to MPA-treated patients in

185 which 56.25% (18/32) required anti-viral treatment (p=0.009) (**Figure 3B**).

186 During the course of CMV infections (all occurring before month 3 post-transplantation),

187 expansion of γδ T cells (**Figure 3C**) and their proportion of late-differentiated effector cells

188 (TEMRA, **Figure 3D**) was more important in mTORi- than MPA-treated patients Moreover,

189 CMV-specific αβ T cell response was also improved since IFNγ measured by QuantiFERON-

190 CMV increased significantly during the course of CMV infection only in mTORi-treated

191 patients (p=0.015) (**Figure 3E**).

192　The proportion of γδ T cells and total CD8+ T cells positive for perforin increased more

193　significantly in mTORi-treated patients, when compared to MPA-treated patients (**Figure 3F**).

194　KLRG1 was expressed on significantly less T cells in mTORi-treated patients (**Figure 3F**).

195　Finally, the decrease of KLRG1+ cells and the increase of perforin+ cells among γδ T cells was

196　directly associated to low viral loads (<999 IU/ml) independently of the immunosuppressive

197　treatment (**Figure 3G**).

198　Altogether, these results show that a better control of CMV infection in mTORi-treated patients

199　compared to MPA-treated patients, was associated with a better expansion and a functional

200　reinforcement of γδ T cells and CD8+ T cells.

201

202　**Long-term *in vitro* treatment of γδ T cells and CMV-specific αβ T cells with therapeutic**

203　**doses of mTORi increase their proliferation and viability**

204　For γδ T cells, comprising more than 95% of TEMRA cells among γδ T cells at day 0

205　(**Supplemental Figure 2A**), PBMC were cultured for 28 days with high doses (1000 IU/ml) of

206　IL-2 (when indicated IL-15 was also added). These conditions allowed TEMRA γδ T cell

207　proliferation despite their late-differentiated phenotype, as shown through CFSE experiments

208　at day 7 of culture (**Supplemental Figure 2B**), and through the strong increase of both

209　percentages (**Supplemental Figure 2C)** and numbers (**Supplemental Figure 3D**) of γδ T cells.

210　To expand CMV-specific αβ T cells, PBMC were cultured with CMV peptides and low dose

211　IL2 (10 IU/ml) (see Methods). Here again, CMV-specific αβ T cells were predominantly

212　TEMRA at day 0 (up to 85%, **Supplemental Figure 2E**).

213　We then used these culture conditions to assess the effect of mTORi, either at dose mimicking

214　anti-proliferative effect, as in oncologic indication (10 nM), or at dose used in

215   immunosuppressive treatment of solid organ transplantation (0.5nM) for γδ T cells, and only at

216   the lowest dose for αβ T cells.

217   As expected and shown in **Figure 4A**, high dose of mTORi inhibited γδ T cell proliferation. By

218   contrast, culture performed with low dose mTORi (0.5 nM) resulted in a better proliferation

219   than in the absence of mTORi. Yet, the dose of 0.5 nM was as potent as that of 10 nM to inhibit

220   S6 phosphorylation (a classical read-out of mTORi action) in TEMRA γδ T cells while not

221   affecting the expression of total S6 protein level (**Figure 4B**). Sequential kinetics analysis of

222   mTORi effect on γδ T cells revealed that the proliferation-promoting action appeared late in the

223   culture (observed at day 21 and day 28) (**Figure 4C**). Interestingly, low and high doses of

224   mTORi were associated with an increased viability of the cells during the late period of the

225   culture (day 28) (**Figure 4D**). Like γδ T cells, low dose mTORi-treated CMV-specific αβ T

226   cells show a better proliferation (**Figure 4E**) and viability (**Figure 4F**). Altogether, these results

227   showed that low dose mTORi improves the proliferation and the viability of TEMRA γδ T cells

228   and CMV-specific αβ T cells consistently with our previous observations in mTORi-treated

229   patients (**Figure 2**)

230

231   **Low dose of mTORi allows to promotes γδ and αβ T cells functional profile**

232   When cultured in the presence of low or high dose of mTORi, a decrease in the proportion of

233   PD-1+ and KLRG1+ γδ T cells was observed while DNAM-1 and TIM-3 receptors were

234   expressed at the same levels (**Figure 5A**). These results suggested that mTORi were able to

235   modify *in vitro* the dysfunctional profile of γδ T cells observed earlier *in vivo* at baseline

236   (**Figure 1**), recapitulating the effect of mTORi observed in patients after transplantation.

237   Second, we tested whether mTORi were acting on transcription factors regulating IFNγ, *i.e.*

238   Tbet, EOMES, Hobit and Blimp-1 as they have been differently involved either in promoting

239 (Hobit and Tbet) or in decreasing (EOMES, Blimp1) IFNγ production (34). We observed that

240 low and high mTORi doses resulted in an increase of Hobit and a decrease of EOMES, which

241 may favor high IFNγ production. Low dose mTORi maintained whereas high dose decreased

242 Tbet expression (**Figure 5B**). Like γδ T cells, CMV-specific αβ T cells treated with a low

243 mTORi dose showed a lower expression of EOMES concomitantly to an increased expression

244 of Hobit (**Figure 5D**), while PD-1+ cells were decreased (**Figure 5C**). Low mTORi dose also

245 reduced the percentage of CD85j+ cells in both T cell subsets (**Supplemental Figure 3**).

246 Third, we wondered if this new phenotype induced by mTORi correlated with a better antiviral

247 function. To this end, γδ T cells were purified after 21 days of culture with mTORi and

248 cocultured with CMV-infected cells for 24 hours. γδ T cells treated with low dose mTORi

249 produced higher amount of IFNγ against CMV-infected cells, which was remarkably not the

250 case when high mTORi dose was used (**Figure 6A**) in accordance with its inhibitory action on

251 Tbet expression (**Figure 5B**). Regarding CMV-specific αβ T cells, PBMC activated with CMV

252 peptides after 7 days of mTORi culture comprised a similar quantity of CMV-specific αβ T

253 cells (IFNγ+CD69+ αβ T cells) (**Supplemental Figure 4**) but an increased IFNγ production

254 (**Figure 6B**) compared to PBMC cultured without mTORi.

255 In summary, our results from *in vitro* cultures show that a low mTORi dose improves functional

256 fitness of γδ T cells and CMV-specific αβ T cells with the reinforcement of CMV-induced IFNγ

257 production. mTORi drive an orientation toward an increased percentage of "Hobit^{high}/

258 EOMES^{low}/PD-1^{low}" cells, previously associated to an efficient antiviral potential (10, 12).

259

260 **Effect of mTORi on TCR engagement and signaling of γδ T cells**

Since it has been previously demonstrated that dysfunctional T cells are hypo-responsive to TCR signaling (35, 36), we took the opportunity that high quantities of γδ T cells (were obtainable (compared to CMV-specific αβ T cells) and we investigated the efficiency of TCR-signaling in γδ T cells treated with or without mTORi in the reactivity against CMV-infected cells (37). TCR neutralization significantly inhibited the production of IFNγ by γδ T cells against CMV-infected cells (**Figure 7A**). This effect was even more pronounced when γδ T cells were pre-incubated with mTORi without viability issue (**Supplemental Figure 5**). These observations suggest that mTORi may amplify the intrinsic ability of γδ T cells to respond to the TCR engagement. In agreement with this hypothesis, IFNγ production by Vδ1 T cells activated using an agonist anti-TCR Vδ1 antibody was increased in mTORi conditions (**Figure 7B**) and in parallel, the internalization of the TCR was significantly more important after 8 hours of activation in mTORi-treated γδ T cells (**Figure 7C**). TCR signaling-induced phosphorylation of the MAP kinases ERK was enhanced in mTORi-treated γδ T cells (**Figure 7D**) while no difference was observed on the phosphorylation of MAPK-38 or of SLP-76 suggesting that mTORi was acting downstream of this very proximal event of TCR signaling (**Supplemental Figure 6**).

Phosphorylation of S6 is also one of the consequences of TCR signaling in T cells and as expected, S6 phosphorylation was decreased at baseline in mTORi-treated γδ T cells (**Figure 7E, left**). However, when normalized according to the basal level of p-S6, the induction of S6 phosphorylation upon TCR triggering was much more important in mTORi-treated than in untreated cells. This was not associated to a feedback activation of Akt (**Supplemental figure 7A and 7B**).

Overall, these results suggest that inhibition of the mTOR pathway conditions γδ T cells to respond more efficiently to an antigenic challenge, as occurs during CMV infection.

285

Discussion

It has been well established that mTORi treatment is associated with a reduction of the incidence of CMV infection in CMV-seropositive organ transplant patients (20, 21), but a mechanistic explanation for this effect has been lacking to date. In the present study, we suggest that a better control of CMV infection in mTORi-treated patients involves a multimodal functional action on both αβ and γδ effector-T cells. In contrast with mTORi immunosuppressive functions on naive allogenic T cells, our overall results indicate that mTORi treatment of specific-effector T cells improves their response to CMV through improving expansion, viability, and restoring their functional phenotype characterized by increased cytotoxic potential, increased IFNγ production and decreased expression of inhibitory check-points.

Our first original observation was to describe at day 0 of the graft a higher proportion of CMV-specific CD8+ and γδ T cells with a dysfunctional phenotype in CMV seropositive patients who were found unable to control CMV infection without antiviral treatment. We characterized this dysfunctional phenotype through the expression of several inhibitory receptors (PD-1, LAG3, TIM3, CD161, CD85j). As usually described, effector memory T cells maintaining after CMV infection have a specific profile with CD45RA reexpression, low PD-1, and high KLRG1, Tbet and Hobit expression without loss of function (6). Here, we described that in cells from CMV seropositive KTR cultured *in vitro*, among effector memory T cells, a percentage of dysfunctional cells expressing PD-1, high level of EOMES, low level of Hobit for the same level of Tbet could be observed. This profile of dysfunctional T cells is usually described in a viral context of a persistent uncontrolled viral load during chronic phases of infection, such as observed with LCMV (38, 39), whereas the effector T cell response described during the CMV

309 infection was called inflationary (5, 6) and correlated to well-controlled low viral load.

310 However, CMV seropositive patients awaiting for kidney graft could be in a relative

311 immunosuppressive state due to the end-stage renal disease (19), to a previous

312 immunosuppressive treatment of their renal disease or during a previous kidney transplantation.

313 This context could lead to a chronic higher level of viral load favoring the emergence of those

314 dysfunctional CMV-effector T cells. Interestingly, documenting the presence of these

315 dysfunctional T cells prior to the introduction of immunosuppressive drug regimen may address

316 an unmet transplant need in transplantation for CMV infection surveillance. Indeed, the

317 identification of a specific biomarker able to predict the risk of CMV reactivation after

318 transplantation in CMV seropositive patients is a long-standing issue. Such a biomarker would

319 be essential to adapt preventive antiviral treatment or CMV monitoring accordingly and avoid

320 useless treatment. Here, PD-1 and CD85j, expressed on both CMV-specific CD8+ and $V\delta2^{neg}$

321 $\gamma\delta$ T cells were the most discriminating markers in R+ patients for predicting severe CMV

322 infection. PD-1 is expressed on T cells following T cell receptor engagement (40) but during

323 chronic antigen exposure, have a sustained expression and contribute to viral clearance failure

324 (41). CD85j is a well-known inhibitory receptor induced on both $\alpha\beta$ (42) and $\gamma\delta$ T cells (43)

325 during CMV infection and is involved in reducing proliferative capability of $\alpha\beta$ T cells (42).

326 Furthermore CD85j is particularly interesting in this context since CMV encodes for UL18, a

327 HLA-like viral protein with the highest affinity for CD85j when compared to other natural

328 ligands (HLA G, HLA A and B) (44).

329 Secondly, we observed that mTORi improved the functional profile of effector T cells together

330 with a lower incidence and severity of CMV infection after transplantation. Indeed, the

331 percentage of PD1+ T cells decreased significantly only in mTORi-treated patients. Moreover,

332 the proportion of CD85j+ cells decreased in CD8+ T cells or remained stable in $\gamma\delta$ T cells in

333 mTORi-treated patients whereas it increased in MPA-treated patients. These results were

confirmed *in vitro* and overall suggest that mTORi treatment may prevent the chronic inhibitory signal transmitted by these two inhibitory immune check points.

Finally, during the course of CMV infection, a better response of T cells to CMV was observed in mTORi-treated patients, as shown by a better expansion of γδ T cells, a more important production of IFNγ when αβ T cells are activated with CMV peptides, a decreased proportion of T cells expressing the inhibitory receptor KLRG1 and an increased proportion of perforin-producing T cells. These changes in T cell profile was directly associated to a better control of CMV independently of the immunosuppressive treatments.

In vitro, low dose of mTORi induced a long-lived cell profile (PD1 low, Tbet high and EOMES low) which was associated with increased proliferation, viability and IFNγ production both on γδ and αβ T cells. Those characteristics were previously associated with improved effector T cells functionality compared to cells expressing high levels of EOMES. and PD-1 and low levels of Tbet (11, 12). Moreover, mTORi also increased the amount of Hobit+ T cells, known to be long-lived effector cells in the context of CMV (8-10).

mTORi could have differential effect on naive and memory cells. In naive cells and notably in allogenic naïve cells, mTORi have been shown to display anti-proliferative effects and mTORi-treated cells present a lower response to activation, underlining the rationale behind using mTORi to prevent graft rejection (24), but in effector T cells, the effect of mTORi have been poorly evaluated. Our *in vitro* results suggest that late-differentiated effector cells present high basal level of p-S6 that limit their capacity for further S6 phosphorylation during activation, that could be involved in their low IFNγ production after TCR engagement. Conversely, when basal level was decreased by mTORi pre-treatment, phosphorylation of S6 was much more inducible during activation in association with a significant increase of IFNγ production. Previous studies reported that effector CD8+ anti-tumor T cells can also display high basal level of p-S6 that associated to high PD-1 expression and mTORi *in vitro* increased their function

359 together with PD-1 decrease (45). Altogether, these results suggest that mTORi are able to

360 condition highly differentiated T cells into a lower level of basal S6 phosphorylation, which

361 allows them to respond much more efficiently upon activation. One aspect that has remained

362 unclear is how mTOR-dependent signals are transmitted to improve T cell responses in effector

363 T cells and why high inhibition of mTOR with high dose lead to T cell inhibition whereas low

364 dose increased T cell function. For example, complete abrogation of mTORC1 signaling via

365 Raptor deletion inhibits mitochondrial biogenesis, while incomplete suppression of mTORC1

366 signaling via Rheb deletion increases mitochondrial synthesis. This could be one clue to explain

367 the differential effect of mTORi doses (46, 47). Moreover, high mTORC1 activation lead to the

368 differentiation in terminal glycolytic effector CD8+ T cells with poor long-term survival that

369 could be restored by rapamycin in mice (47).

370 Finally, we also observed that the improvement of IFNγ production by mTORi-treated γδ T

371 cells activated by CMV-infected cells was TCR-dependent. Moreover, TCR signaling

372 improvement was also highlighted by assessing direct TCR activation and p-ERK

373 phosphorylation. This is of particular interest since late effector memory T cells during chronic

374 infection are mostly dependent on TCR signaling rather than cytokine signals to persist (48,

375 49). We can hypothesize that chronic antigen activation leads to increased basal mTOR levels

376 in T cells, hampering their capacity to respond properly during a new viral challenge.

377 Decreasing the basal level of mTOR pathway activation could sensitize effector T cells to better

378 respond to a new TCR activation.

379 Altogether, those findings have highlighted the involvement of dysfunctional T cells in CMV

380 infection risk after transplantation. CD85j and PD-1 expression offers the most promising

381 perspective in term of markers to better manage prevention strategies in CMV seropositive

382 patients. We believe that these results provide a clinical and mechanistic understanding of

383 mTORi effect on the restoration of CMV-specific late effector T cells, leading to a better control

384 of CMV after transplantation. These new insights could guide better use of mTORi in CMV

385 seropositive patients leading to improved outcomes for the most frequent opportunistic

386 infection in solid organ transplantation.

387 .

Materials and Methods

Sample and Patients

Samples were obtained from 79 CMV seropositive kidney transplant recipients who were enrolled at day 0 of their kidney transplantation in an ancillary study of a phase IV clinical trial which compared the incidence of CMV DNAemia in patients receiving mTORi (everolimus, n=27) or mycophenolic acid (MPA, n=44). The protocol was approved by an independent ethics committee (CPP number: 2013/57) and registered in the ClinicalTrials.gov database (NCT02328963). All subjects signed a written informed consent before enrollment. CMV serostatus for each kidney transplant recipients had been determined the day of the graft. $V\delta2^{neg}$ $\gamma\delta$ T cells immunophenotyping was performed at week 1, week 2 and week 24 post-transplantation. Peripheral blood mononuclear cells (PBMC) were isolated and frozen at day 0, month 1, month 3, month 6 and month 12 post-transplantation. The course of CMV infection was preemptively monitored by longitudinal whole blood quantitative nucleic acid testing (QNAT) for CMV viral load every week from day 0 to month 3, then at month 4, 5, 6, 9 and 12, as previously described (50). The results were all given in IU/ml, calibrated with the International Standard WHO (51). The lower limit of quantification was 1000 IU/ml and of detection 250IU/ml; consequently, when the result was "weak positive", we put 999IU/ml. CMV DNAemia was defined as a positive QNAT (52). Antiviral treatment with ganciclovir was started at the time of the first >2000 IU/mL positive CMV QNAT.

Ex vivo **phenotyping of $\gamma\delta$ T cells and CMV-specific CD8+ T cells.**

For complete reference of antibodies, see supplemental Methods.

Flow cytometry phenotyping of patients' $V\delta2^{neg}$ $\gamma\delta$ T cells was performed on whole blood as previously described (53) using the following monoclonal antibodies: anti-CD45-APC, anti-panδ-PE, anti-Vδ2-FITC, anti-CD27 Pe-Cy7 and anti-CD45RA-ECD and with the Lysing

413   Solution IOTest®3 10X Concentrate (Beckman). The samples were processed on a NAVIOS

414   flow cytometer (Beckman coulter).

415   One million frozen PBMC were used for each multi-color flow cytometry analysis, with the

416   same viability marker FVS575, the same monoclonal antibodies against CD3 (PerCP), Vδ2

417   (PC-7), pan-delta (PE or APC), CD8 (either BV510 or PE-texas-red) and IFN-γ (BV786) and

418   CD69 (AF-700) for the staining of CMV-specific CD8+ T cells. Then, the staining included

419   either CD45RA (FITC), and CD27 (BV786); or CD85j (FITC), CD161 (BV650), CD16

420   (BV786), and KLRG1 (PE-Vio 615); or granulysin (FITC), perforin (APC) and granzyme

421   (BV421); or PD-1 (BV650), TIM-3 (BV711), LAG3 (BV421), and DNAM-1 (BV786).

422   PBMC   stained   for   intracellular   markers   were   permeabilized   and   fixed   using

423   Fixation/Permeabilization Solution (BD biosciences) , then resuspended in PBS supplemented

424   with 0.1% bovine serum albumin and 2mM EDTA. Stained cells were processed on the BD

425   LSRFortessa (BD biosciences).

426   *Ex vivo* **quanti-FERON-CMV.**

427   QuantiFERON-CMV V (n°0350-0201, Qiagen) was performed as previously described (54)

428   from frozen plasma and read with the QUANTA-Lyser® 2 Inova Diagnostics; at day 14 (32

429   mTORi-treated and 43 MPA-treated patients) and month 6 post transplantation (24 mTORi-

430   treated and 39 MPA-treated patients). Results were analyzed with the CMV v3.03 software.

431

432   **PBMC cultures for T cell expansions.**

433   For γδ T cell cultures, PBMC were cultured in RPMI medium supplemented with 2mM

434   glutamine, 10% human serum, 1000 IU/ml recombinant human IL-2 (rIL2, n°200-02), (all from

435   Peprotech, France) for 28 days with 0; 0.5 or 10nM of the mTORi everolimus (HY-102018,

436 Medchemexpress EU). In some experiments 10 ng/ml recombinant human IL-15 (rIL15, n°200-

437 15) was added.

438 For CMV-specific αβ T cell cultures, PBMC were cultured in RPMI medium-2mM glutamine-

439 10% human serum-10 IU/ml recombinant human IL-2 for 16 days with 0 or 0.5nM of

440 everolimus. 0.6 nmol/ml of peptivator CMV pp65 (130-093-438, Miltenyi) was added at day 0

441 and day 9.

442

443 **Proliferation and viability assay for γδ and CMV-specific αβ T cells**

444 Proliferation within TEMRA cells was first assessed by CFSE assay. PBMC were labelled with

445 5 μM 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, n°V12883, eBioscience,

446 France) and cultured either RPMI medium supplemented with 10% human serum (not

447 stimulated) with or without 1000 U/ml rIL-2 and 10 ng/ml rIL-15 (stimulated). At day 7 of

448 culture, $2x10^5$ cells were incubated with monoclonal antibodies anti-pan TCRδ (PE), TCRVδ2-

449 PC-7 and anti-CD3 (BV510), anti-CD45RA (BV786), CD27 (BV650). Then, cells were

450 washed, were processed on the BD LSRFortessa flow cytometer.

451 Proliferation of TEMRA Vδ2^{neg} γδ T cells was also assessed by counting on a Neubauer cell

452 counting chamber and phenotyping at day 0, 7, 14, 21 and 28: $2x10^5$ cells from the IL2

453 (+/- IL15) cultures were washed and stained with monoclonal antibodies anti-CD3 (V450),

454 CD27 (APC) and CD45RA (FITC) antibodies, panδ (PE), Vδ2 (PC7) and all cells were

455 processed with a BD Canto II cytometer (BD biosciences).

456 For CMV-specific αβ T cell proliferation assessment, PBMC were incubated in 96 well plates

457 with 150μL of the previously described media. At day 9, 0.6 nmol/mL of peptivator CMV pp65

458 was added with a protein transport inhibitor (n°554724, BD biosciences) overnight at 37°C and

459 then cells were stained with anti-CD3 (V450), CD27 (BV650), CD45RA (FITC) (BD

460 biosciences), pan-αβ (APC), CD69-PE monoclonal antibodies. Fixation-permeabilization was

461   performed and the cells were finally stained with an anti-IFNγ (BV786) antibody. CMV-

462   specific αβ T cells were gated as double positive cells for IFNγ and CD69 (55).

463   In order to obtain absolute numbers, percentages of CMV-specific αβ and Vδ2^{neg} T cells among

464   PBMC in culture were multiplied by the total PBMC count at the same time. Fold increases

465   (FI) were obtained as the ratio of Vδ2^{neg} T cell counts at day 14, 21 and 28, or CMV-specific

466   αβ T cell counts at day 9 and 16, to count at day 0.

467   Viability was measured by incubating $2x10^5$ cultured PBMC with the viability marker FVS575,

468   at day 14, 21 and 28 days of culture for Vδ2^{neg} T cells, and at day 9 and 16 for CMV-specific

469   αβ T cells. PBMC were processed on the BD LSRFortessa flow cytometer.

470

471   **Staining of co-receptors and transcription factors for γδ and CMV-specific αβ T cells**

472   For Vδ2^{neg} γδ T cells, $5x10^5$ PBMC at day 21 of culture were washed, and incubated with either

473   the viability marker FVS575, then with antibodies anti-CD3 (BV510), PD-1 (BV650), TIM3

474   (BV711), DNAM-1 (BV786), panδ (PE), Vδ2 (PC7) and KLRG1 (FITC); or the viability

475   marker FVS780, then with antibodies anti-CD3 (BV510), Vδ2 (pacific blue) and either panδ

476   (APC) with Blimp1 (PE), T-bet (PC5.5), or EOMES (Pe-Cy7) alone or panδ (PE) with Hobit

477   (alex-fluor 647) (BD bioscience). After extracellular staining, PBMC were permeabilized with

478   the FOXP3 transcription factor staining buffer (Fisher scientific). For CMV-specific αβ T cells,

479    $5x10^5$ PBMC cultured as described above in 96-wells plates, were first stained with the viability

480   marker FVS575, then either with antibodies anti-PD-1 (BV650), KLRG1 (FITC), pan-

481   αβ (APC), CD3 (V450), CD69 (PE) antibodies and intracellular staining with anti-IFNγ

482   (BV786) or anti-pan-αβ (PE), CD3 (V450), CD69 (Alexa Fluor 700) antibodies , then anti-

483   EOMES (PC7), Hobit (alexa-fluor 647) and IFNγ (BV786) antibodies. The totality of PBMC

484   was processed by the BD LSRFortessa flow cytometer.

485

Flow cytometry evaluation of S6 and Akt phosphorylation.

2.10^5 cells were isolated from the Vδ2^{neg} γδ expansion cultures at day 21, washed and incubated in 400μL of RPMI (2mM glutamine, 8% FCS alone or with everolimus) and activated with an anti-Vδ1 TCR mAb (10μg/ml) for 0-30min-2 and 4 hours for Akt and 0-2-4-6 hours for S6 in a 48-wells plate. Then, cells were washed with PBS-EDTA2mM-azide 0.09%-FCS 2% (Phosphoflow buffer), stained on ice in 50μL of Phosphoflow buffer and incubated with monoclonal antibodies anti-CD3 (V450), Vδ2 (FITC) and panδ (APC) for 10 min (min). Intracellular stainings for phosphorylated proteins were done as previously described (56) with either anti-pS6 (PeCy7), S6 (PE), pAkt T308 (PE), pAkt S473 (FITC), Akt (PE) and processed by Canto II.

496

Staining of pSLP-76, p-ERK and p38 of γδ T cells

At day 21 of culture with rIL2, Vδ2^{neg} γδ T cells were purified after magnetic negative sorting with pan T cell isolation kit; αβ–biotin and anti-biotin (Miltenyi Biotec), Vδ2 FITC (Beckman) and anti-FITC (Miltenyi Biotec). Purity was controlled by staining 50 000 sorted cells with anti-CD3 (V450), panδ (PE), and Vδ2 (PC7) with a 85-96% purity. 50 000 cells were activated in 50μL of RPMI (2mM glutamine, 8%FCS without or without everolimus) with 10μg/ml of purified UCHT1 (Beckman) for 0-1/2-4/10 min for pSLP76 (AF 647) and pERK (FITC) staining and 0/10/15 min for MAPK-p38 (AF 647) staining. Intracellular staining was subsequently performed as previously described (56) and cells were processed on Canto II.

506

Preparation of CMV-infected fibroblasts

Human foreskin fibroblasts (HFF, kindly provided by Dr H. Rezvani, INSERM, U1035, Bordeaux), grown in Dulbecco's Modified Eagle Medium (DMEM) containing 8% FCS and

510 2mM glutamine, were infected with the TB42/E strain of human CMV at a multiplicity of

511 infection (MOI) of 0.1. After virus adsorption overnight at 37°C, cells were washed and covered

512 with fresh growth medium. Co-cultures were performed when cytopathic effects were ≥90%, 4

513 days after infection. Non infected (NI) cells grown in parallel were mock-infected using

514 medium alone.

515

516 **IFNγ production assay by ELISA**

517 After 21 days of culture with rIL-2 and rIL-15, Vδ2^{neg} γδ T cells were negatively purified by

518 magnetic sorting as described above and were checked for purity. 50.10^4 cells were incubated

519 *per* 96 wells-plate with RPMI 8% FCS, 2mM glutamine and 50ng/ml rIL18 (MBL international,

520 B003-5, Massachusetts-US) (see (57)) with or without everolimus alone, or with either non-

521 infected or CMV-infected fibroblasts during 24 hours at 37°C. For CMV-specific αβ T cells,

522 PBMC were cultured as described above but without pp-65 stimulation and after 7 days, cells

523 were incubated alone or with peptivator CMV- pp65 0.6nmol/mL or PMA 25ng/ml/ionomycine

524 1µg/ml for 37°C during 24 hours. An extra well was added to perform an IFNγ and CD69

525 staining after overnight pp65 stimulation with a protein transport inhibitor.

526 Then, the supernatants were collected for IFN-γ ELISA operated as described in the kit (Human

527 IFN-γ ELISA development kit, Mabtech, n°3420-1H-6).

528

529 **Statistics**

530 The Mann-Whitney U test, the χ^2 test or the Fisher-test, the unpaired t test were used when

531 appropriate. $p<0.05$ was considered statistically significant. Alternatively, paired-t test was

532 used for paired data. Analyses were performed with conventional statistical methods using

533 GraphPad Prism. Figures were obtained with FlowJo software (V.10) and GraphPad Prism**.**

534

Author contributions

The authors have declared that no conflict of interest exists.

HK was involved in designing research studies, conducting experiments, acquiring data, analyzing data, and writing the manuscript. NY, GM, MJN, AT, AZ, VP, SL, XG, IG , BP, KH were involved in acquiring and analyzing data, and writing the manuscript. LC, IP, RT, RD, MMM, MC were involved in analyzing data and writing the manuscript. PM and JDM were involved in designing research studies, in analyzing data, and writing the manuscript.

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706

707

Figure legends

Figure 1 Vδ2^{neg} γδ T cells and CD8+ T cells express inhibitory receptors at baseline in patients with severe CMV infections

Vδ2^{neg} γδ T cells and total CD8+ T cells were analyzed for their expression of CD27 and CD45RA (A) and their co-stimulatory and co-inhibitory receptors (B), in all CMV seropositive MPA treated patients (n=21) (A-B) or separating patients with well-controlled CMV (n=12) versus patients severe CMV (n=9) (C). Finally, phenotypes were extended to CMV-specific CD8+ T cells in an internal validation cohort of patients with well-controlled CMV (n=5) versus patients with severe CMV (n=7) (D). Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation, $0.05>p>0.01$*; **$0.01>p>0.001$; ***$p<0.001$; as determined by the Mann-Withney U test.

Figure 2 High percentage of functional T cells in mTORi-treated patients correlated to a lower incidence of CMV infection

A. Incidence of CMV DNAemia in 27 mTORi-treated patients and in 44 patients treated with mycophenolic acid (MPA) at month 12 post-transplantation.

B.C. Whole blood staining of Vδ2^{neg} γδ T cells and their expression of CD27 and CD45RA. Frequencies of Vδ2^{neg} γδ T cells among T cells (B), of TEMRA (CD27 neg CD45RA pos) and naive (CD27 hi CD45RA pos) cells among Vδ2^{neg} γδ T cells (C) at day 14 post-transplantation. Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation.

D. CMV-specific αβ T cells were analyzed with QuantiFERON-CMV (IU/mL) at day 14 post-transplantation. Each symbol represents an individual donor, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation.

732 E. Proportions among Vδ2^neg γδ T cells (left), among total CD8+ T cells (middle) and CMV-

733 specific CD8+ T cells (right) of CD85j+, perforin+, KLRG1+ and PD-1+ cells, compared

734 between day 0 (d0) and month 1 (m1) post-transplantation in mTORi (n=8) and MPA (n=7)-

735 treated patients. Each symbol represents an individual donor.

736 F. Difference of CD85j and PD-1+ cell percentage between month 1 and day 0 (value m1- value

737 day0) was calculated and compared in patient groups with a post-transplantation CMV viral

738 load > or <1000IU/ml (including 0IU/ml). $0.05 > p > 0.01$*; **$0.01 > p > 0.001$; ***$p < 0.001$; as

739 determined for all unpaired data by the Mann-Withney U test and for paired data by Wilcoxon

740 test.

741

742 **Figure 3 Better response of T cells in mTORi-treated patients than in MPA-treated**

743 **patients correlated to a lower severity of CMV infection**

744 A. Maximal CMV viral load (IU/ml) in mTORi (n=7) and MPA (n=32)-treated patients. Each

745 symbol represents CMV viral load value for an individual patient; large horizontal lines indicate

746 the mean.

747 B. Proportion of MPA (n=38) and mTORi (n=7) treated patients with CMV DNAemias,

748 $0.05 > p > 0.01$*; as determined by the Fisher's exact test.

749 C.D. Whole blood staining of Vδ2^neg γδ T cells and their expression of CD27 and CD45RA.

750 Proportion of Vδ2^neg γδ T cells among T cells (C) and of TEMRA (CD27 neg CD45RA pos)

751 among Vδ2^neg γδ T cells at week 1, 2, 12 and 24 post-transplantation in mTORi (n=7) and MPA

752 (n=32)-treated patients. Each symbol represents the median-value for each group of patients

753 associated with interquartile ranges.

754 E. Quantiferon-CMV (IU/ml) at week one and week 24 in mTORi(n=7) and MPA-treated

755 patients (n=22) with CMV DNAemia post transplantation. Each symbol represents an

756 individual donor.

757   F. Proportions among Vδ2^{neg} γδ T cells (left) and among total CD8+ T cells (right) of perforin+

758   and KLRG1+ cells, compared between day 0 and during CMV DNAemia (either month 1 or

759   month 3) in mTORi (n=7) and MPA (n=7) treated patients. Each symbol represents an

760   individual donor.

761   G. Difference of KLRG1 and perforin+ Vδ2^{neg} γδ T cell percentages between month 1 and day

762   0 were calculated and compared in patient groups with a post-transplantation CMV viral load

763   > or = 1000 IU/ml ns, not significant, $0.05>p>0.01$*; **$0.01>p>0.001$ as determined for paired-

764   data by Wilcoxon test and for unpared data by Mann-Withney U test.

765

766   **Figure 4 Impact of long-term *in vitro* mTORi treatment on proliferation and viability of**

767   **Vδ2^{neg} γδ T cells and CMV-specific αβ T cells**

768   PBMC from CMV seropositive KTR were incubated with IL-2 with or without IL-15 for Vδ2^{neg}

769   γδ T cells and IL-2 alone for CMV-specific αβ T cells and with indicated doses of everolimus.

770   Proliferation and viability of Vδ2^{neg} γδ T cells at day 14, 21 and 28 of culture and of CMV-

771   specific αβ T cells at day 9 and 16 of culture were performed.

772   A. Representative donor for Vδ2^{neg} γδ T cells proliferation.

773   B. Representative flow cytometry staining of S6 and phospho-S6 (p-S6) among Vδ2^{neg} γδ T

774   cells at day 14 of culture.

775   C. Proliferation of Vδ2^{neg} γδ T cells, represented as fold increases normalized to culture with

776   medium alone, at day 14 (everolimus 0.5nM, n=13; everolimus 10nM, n=6), 21 (everolimus

777   0.5nM, n=15; everolmus 10nM, n=8) and 28 (everolimus 0.5nM, n=12; everolimus 10nM,

778   n=6).

779   D. Vδ2^{neg} γδ T cell viability tested by flow cytometry live-dead staining (n=5).

780   E. Proliferation of CMV specific $\alpha\beta$ T cells, represented as fold increases normalized to culture

781   with medium alone (everolimus 0.5nM, n=5)

782   F. CMV-specific $\alpha\beta$ T cells viability tested by flow cytometry live-dead staining (n=5).

783   For C, D, E and F, each symbol represents an individual donor. ns, not significant,

784   0.05>p>0.01*; **0.01>p>0.001; ***0p<0.001 as determined by Wilcoxon test.

785

786   **Figure 5 Low dose mTORi improves the functional profile of V$\delta2^{neg}$ $\gamma\delta$ T cells and CMV-**

787   **specific $\alpha\beta$ T cells**

788   V$\delta2^{neg}$ $\gamma\delta$ T cells (after 21 days) and CMV-specific $\alpha\beta$ T cells (after 16 days) were analyzed

789   after *in vitro* culture of PBMC from CMV-seropositive KTR with or without everolimus.

790   Frequencies of PD-1, KLRG1, DNAM-1 and TIM-3 (A) and Tbet, Blimp-1, EOMES and Hobit

791   (B) among V$\delta2^{neg}$ $\gamma\delta$ T cells for one representative donor (top) and for 5 donors (bottom).

792   Frequencies of PD-1 and KLRG1 (C) and of EOMES and Hobit (D) among CMV specific $\alpha\beta$

793   T cells for one representative donor (left) and 5 donors (right). Each symbol represents an

794   individual donor. ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by

795   Wilcoxon test.

796

797   **Figure 6 Low dose mTORi improves V$\delta2^{neg}$ $\gamma\delta$ T cells and CMV-specific $\alpha\beta$ T cells**

798   **response to CMV**

799   A.V$\delta2^{neg}$ $\gamma\delta$ T cells were purified and cultured in medium alone or with non-infected (NI) or

800   CMV-infected fibroblasts during 24 hours and ELISA IFNγ was performed (n= 4 donors).

801   B. PBMC from CMV seropositive donors were cultured during 7 days then maintained in

802   medium alone or stimulated with 0.6 nmol/l of pp65 peptivator during 24 hours, and ELISA

803   IFNγ was performed (n=5). Each symbol represents an individual donor and in Figure A, large

804   horizontal lines indicate the mean and small horizontal lines indicate the standard deviation; ns,

805   not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

806

807   **Figure 7 Effect of mTORi on TCR engagement and signaling of Vδ2^{neg} γδ T cells**

808   A. Vδ2^{neg} γδ T cells were purified and cultured in medium alone, with non-infected (NI) or

809   CMV-infected fibroblasts with or without a blocking anti-CD3 mAb (10μg/ml) during 24 hours

810   and ELISA IFNγ) was performed (n=4 donors).

811   B. C. Vδ2^{neg} γδ T cells among total PBMC were specifically stimulated via their TCR, by an

812   anti-Vδ1 mAb (10 μg/ml), for 2, 4 and 6 hours, then ELISA IFNγ was performed (B), and cells

813   were stained for gamma-delta TCR downregulation analysis by flow cytometry (C) (n=4

814   donors).

815   D. Vδ2^{neg} γδ T cells were purified and stimulated with an anti-CD3 antibody (UCHT1,

816   10μg/ml) for 0, 1, 2, 4 and 7 min. Erk 1/2 phosphorylation was measured by flow cytometry

817   (one representative donor, right; in 4 donors (left).

818   E. Vδ2^{neg} γδ T cells among total PBMC were specifically stimulated via their TCR, by an anti-

819   Vδ1 mAb (10μg/ml) for 2, 4 and 6 hours and S6 phosphorylation was measured by flow

820   cytometry. Basal levels before stimulation are represented for 7 donors (left) and activation

821   kinetics normalized on basal level for each condition (0 and 0.5 nM everolimus) (right) for 4

822   donors are represented. Each symbol represents an individual donor, large horizontal lines

823   indicate the mean and small horizontal lines indicate the standard deviation in Figure A and E

824   (left). Each symbol represents the median of the results for 4 donors and small horizontal lines

825   represent the ranges in Figure B, C, D and E (right). ns, not significant, $0.05 > p > 0.01$*;

826   **$0.01 > p > 0.001$; as determined by Wilcoxon test.

827

828

829

Figure 1

Figure 1 Vδ2^{neg} γδ T cells and CD8+ T cells express inhibitory receptors at baseline in patients with severe CMV infections

Vδ2^{neg} γδ T cells and total CD8+ T cells were analyzed for their expression of CD27 and CD45RA (A) and their co-stimulatory and co-inhibitory receptors (B), in all CMV seropositive MPA treated patients (n=21) (A-B) or separating patients with well-controlled CMV (n=12) versus patients severe CMV (n=9) (C). Finally, phenotypes were extended to CMV-specific CD8+ T cells in an internal validation cohort of patients with well-controlled CMV (n=5) versus patients with severe CMV (n=7) (D). Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation, 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined by the Mann-Withney U test.

Figure 2 High percentage of functional T cells in mTORi-treated patients correlated to a lower incidence of CMV infection

A. Incidence of CMV DNAemia in 27 mTORi-treated patients and in 44 patients treated with mycophenolic acid (MPA) at month 12 post-transplantation. B.C. Whole blood staining of Vδ2^{neg} γδ T cells and their expression of CD27 and CD45RA. Frequencies of Vδ2^{neg} γδ T cells among T cells (B), of TEMRA (CD27 neg CD45RA pos) and naive (CD27 hi CD45RA pos) cells among Vδ2^{neg} γδ T cells (C) at day 14 post-transplantation. Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation. D. CMV-specific αβ T cells were analyzed with QuantiFE-RON-CMV (IU/mL) at day 14 post-transplantation. Each symbol represents an individual donor, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation. E. Proportions among Vδ2^{neg} γδ T cells (left), among total CD8+ T cells (middle) and CMV-specific CD8+ T cells (right) of CD85j+, perforin+, KLRG1+ and PD-1+ cells, compared between day 0 (d0) and month 1 (m1) post-transplantation in mTORi (n=8) and MPA (n=7)-treated patients. Each symbol represents an individual donor. F. Difference of CD85j and PD-1+ cell percentage between month 1 and day 0 (value m1- value day0) was calculated and compared in patient groups with a post-transplantation CMV viral load > or <1000IU/ml (including 0IU/ml). 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined for all unpaired data by the Mann-Withney U test and for paired data by Wilcoxon test.

Figure 3
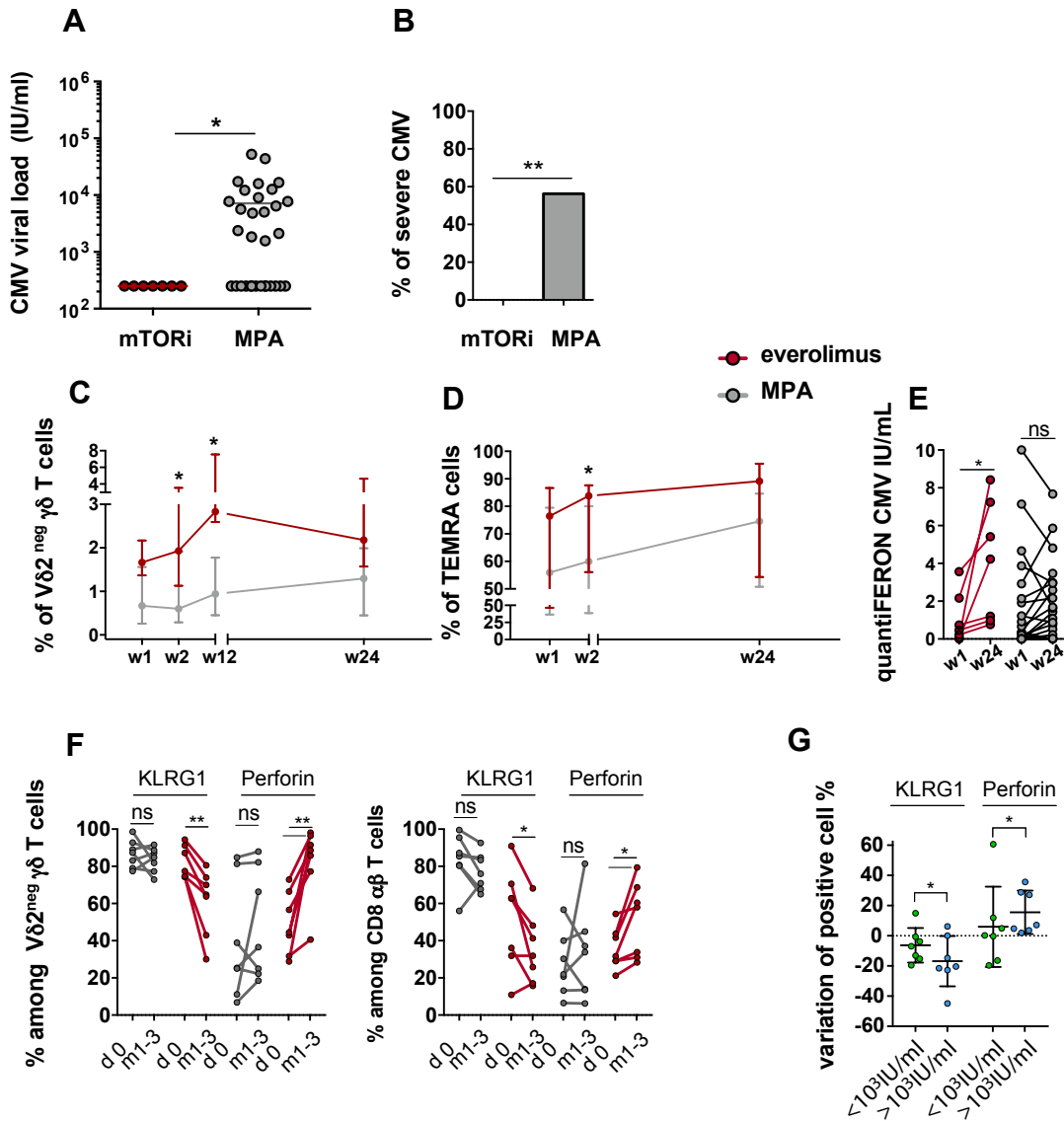
Figure 3 Better response of T cells in mTORi-treated patients than in MPA-treated patients correlated to a lower severity of CMV infection

A. Maximal CMV viral load (IU/ml) in mTORi (n=7) and MPA (n=32)-treated patients. Each symbol represents CMV viral load value for an individual patient; large horizontal lines indicate the mean. B. Proportion of MPA (n=38) and mTORi (n=7) treated patients with CMV DNAemias, 0.05>p>0.01*; as determined by the Fisher's exact test. C.D. Whole blood staining of Vδ2^{neg} γδ T cells and their expression of CD27 and CD45RA. Proportion of Vδ2^{neg} γδ T cells among T cells (C) and of TEMRA (CD27 neg CD45RA pos) among Vδ2^{neg} γδ T cells at week 1, 2, 12 and 24 post-transplantation in mTORi (n=7) and MPA (n=32)-treated patients. Each symbol represents the median-value for each group of patients associated with interquartile ranges. E. Quantiferon-CMV (IU/ml) at week one and week 24 in mTORi(n=7) and MPA-treated patients (n=22) with CMV DNAemia post transplantation. Each symbol represents an individual donor. F. Proportions among Vδ2^{neg} γδ T cells (left) and among total CD8+ T cells (right) of perforin+ and KLRG1+ cells, compared between day 0 and during CMV DNAemia (either month 1 or month 3) in mTORi (n=7) and MPA (n=7) treated patients. Each symbol represents an individual donor. G. Difference of KLRG1 and perforin+ Vδ2^{neg} γδ T cell percentages between month 1 and day 0 were calculated and compared in patient groups with a post-transplantation CMV viral load > or = 1000 IU/ml ns, not significant, 0.05>p>0.01*; **0.01>p>0.001 as determined for paired-data by Wilcoxon test and for unpared data by Mann-Withney U test.
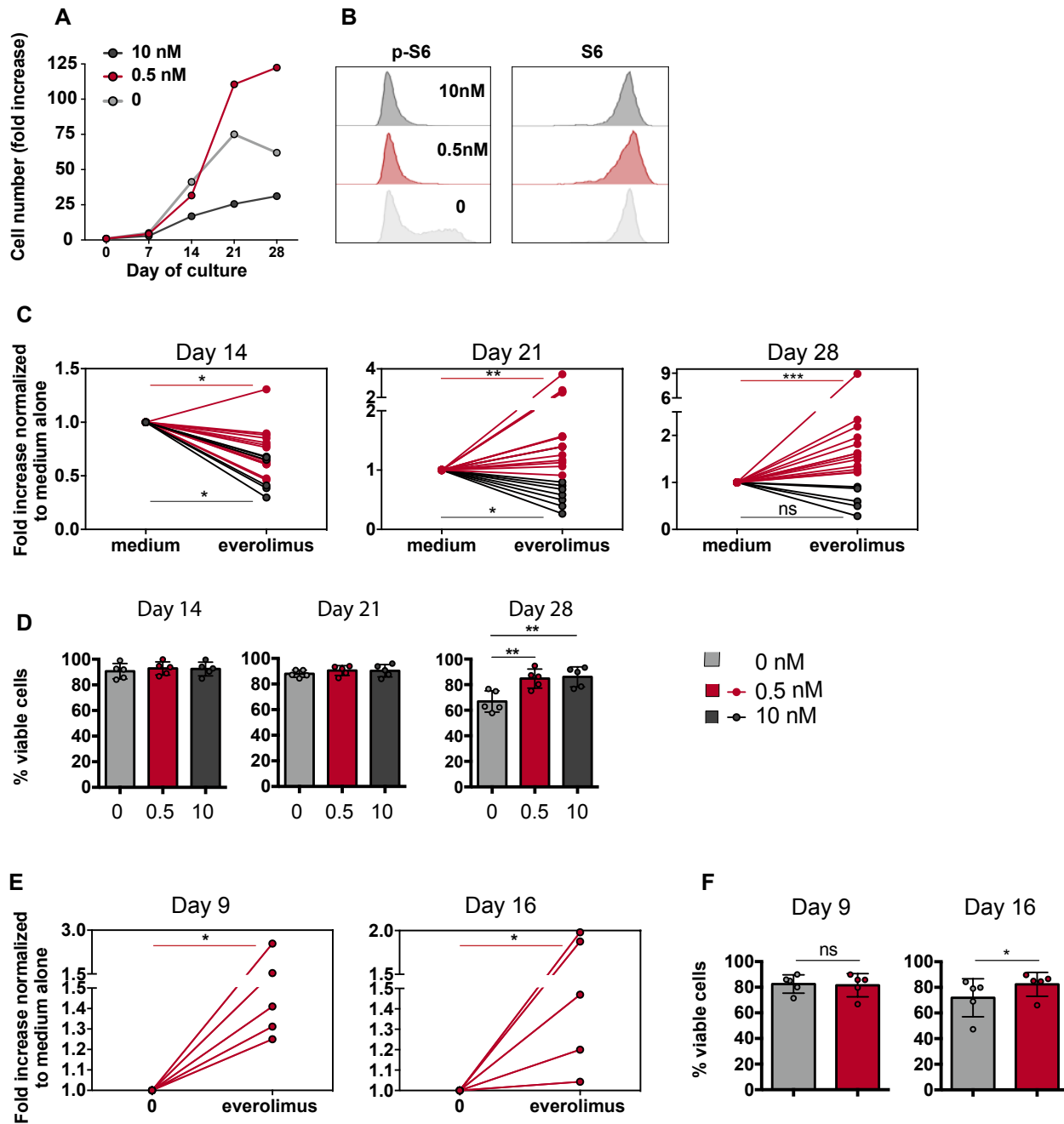
Figure 4

Figure 4 Impact of long-term *in vitro* mTORi treatment on proliferation and viability of Vδ2^neg γδ T cells and CMV-specific αβ T cells

PBMC from CMV seropositive KTR were incubated with IL-2 with or without IL-15 for Vδ2^neg γδ T cells and IL-2 alone for CMV-specific αβ T cells and with indicated doses of everolimus. Proliferation and viability of Vδ2^neg γδ T cells at day 14, 21 and 28 of culture and of CMV-specific αβ T cells at day 9 and 16 of culture were performed.

A. Representative donor for Vδ2^neg gδ T cells proliferation. B. Representative flow cytometry staining of S6 and phospho-S6 (p-S6) among Vδ2^neg γδ T cells at day 14 of culture. C. Proliferation of Vδ2^neg γδ T cells, represented as fold increases normalized to culture with medium alone, at day 14 (everolimus 0.5nM, n=13; everolimus 10nM, n=6), 21 (everolimus 0.5nM, n=15; everolmus 10nM, n=8) and 28 (everolimus 0.5nM, n=12; everolimus 10nM, n=6). D. Vδ2^neg γδ T cell viability tested by flow cytometry live-dead staining (n=5). E. Proliferation of CMV specific αβ T cells, represented as fold increases normalized to culture with medium alone (everolimus 0.5nM, n=5). F. CMV-specific αβ T cells viability tested by flow cytometry live-dead staining (n=5). For C, D, E and F, each symbol represents an individual donor. ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; ***0p<0.001 as determined by Wilcoxon test.

Figure 5
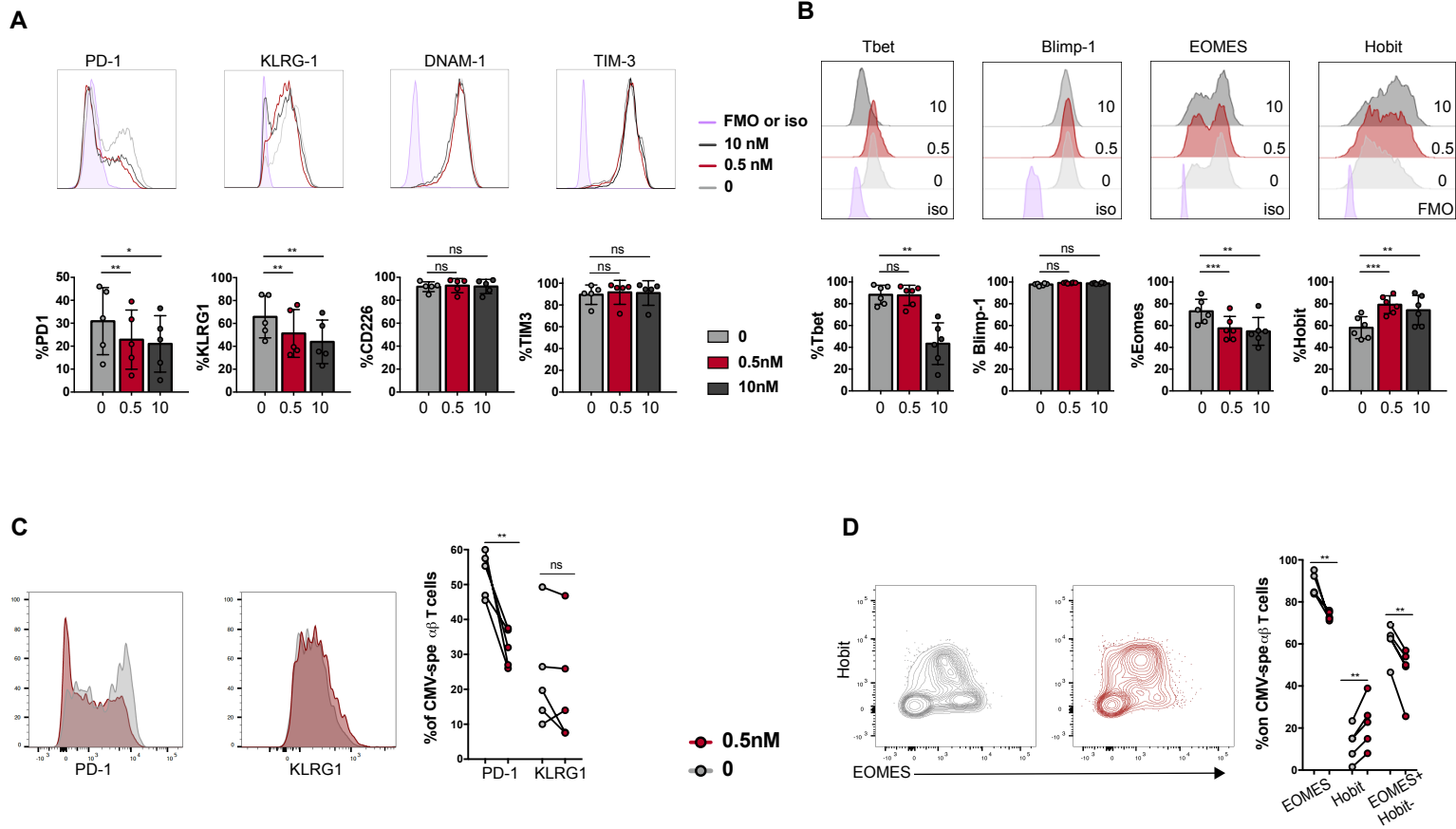
Figure 5 Low dose mTORi improves the functional profile of Vδ2^{neg} γδ T cells and CMV-specific αβ T cells

Vδ2^{neg} γδ T cells (after 21 days) and CMV-specific αβ T cells (after 16 days) were analyzed after *in vitro* culture of PBMC from CMV-seropositive KTR with or without everolimus.

Frequencies of PD-1, KLRG1, DNAM-1 and TIM-3 (A) and Tbet, Blimp-1, EOMES and Hobit (B) among Vδ2^{neg} γδ T cells for one representative donor (top) and for 5 donors (bottom).

Frequencies of PD-1 and KLRG1 (C) and of EOMES and Hobit (D) among CMV specific αβ T cells for one representative donor (left) and 5 donors (right). Each symbol represents an individual donor. ns, not significant, $0.05 > p > 0.01$*; **$0.01 > p > 0.001$; as determined by Wilcoxon test.

Figure 6
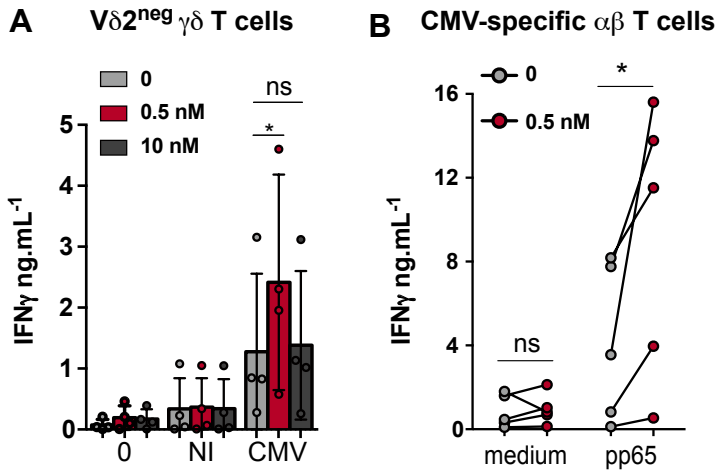
Figure 6 Low dose mTORi improves Vδ2^{neg} γδ T cells and CMV-specific αβ T cells response to CMV

A.Vδ2^{neg} γδ T cells were purified and cultured in medium alone or with non-infected (NI) or CMV-infected fibroblasts during 24 hours and ELISA IFNγ was performed (n= 4 donors).

B. PBMC from CMV seropositive donors were cultured during 7 days then maintained in medium alone or stimulated with 0.6 nmol/l of pp65 peptivator during 24 hours, and ELISA IFNγ was performed (n=5). Each symbol represents an individual donor and in Figure A, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation; ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

Figure 7
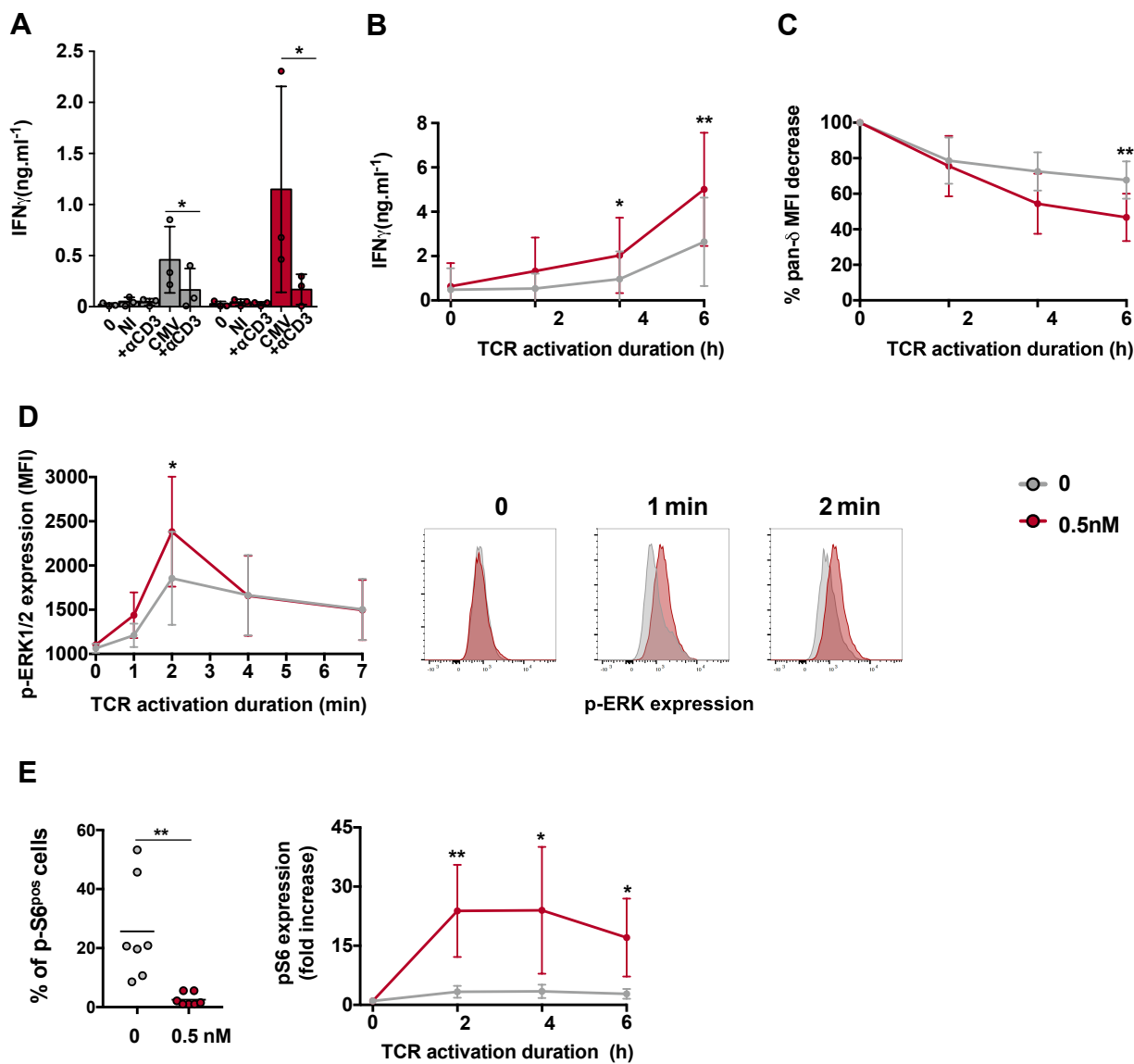
Figure 7 Effect of mTORi on TCR engagement and signaling of Vδ2^{neg} γδ T cells

A. Vδ2^{neg} γδ T cells were purified and cultured in medium alone, with non-infected (NI) or CMV-infected fibroblasts with or without a blocking anti-CD3 mAb (10μg/ml) during 24 hours and ELISA IFNγ) was performed (n=4 donors).

B. C. Vδ2^{neg} γδ T cells among total PBMC were specifically stimulated via their TCR, by an anti-Vδ1 mAb (10 μg/ml), for 2, 4 and 6 hours, then ELISA IFNγ was performed (B), and cells were stained for gamma-delta TCR downregulation analysis by flow cytometry (C) (n=4 donors).

D. Vδ2^{neg} γδ T cells were purified and stimulated with an anti-CD3 antibody (UCHT1, 10μg/ml) for 0, 1, 2, 4 and 7 min. Erk 1/2 phosphorylation was measured by flow cytometry (one representative donor, right; in 4 donors (left).

E. Vδ2^{neg} γδ T cells among total PBMC were specifically stimulated via their TCR, by an anti-Vδ1 mAb (10μg/ml) for 2, 4 and 6 hours and S6 phosphorylation was measured by flow cytometry. Basal levels before stimulation are represented for 7 donors (left) and activation kinetics normalized on basal level for each condition (0 and 0.5 nM everolimus) (right) for 4 donors are represented. Each symbol represents an individual donor, large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation in Figure A and E (left). Each symbol represents the median of the results for 4 donors and small horizontal lines represent the ranges in Figure B, C, D and E (right). ns, not significant, 0.05>p>0.01*; **0.01>p>0.001; as determined by Wilcoxon test.

1 **Supplementary materials**

2 **Supplemental Methods**

3 **Immunosuppressive regimen of patients**

4 Induction therapy was anti-IL2RA (20mg on day 0 and day 4) (SIMULECT®, Novartis Pharma

5 SAS) and Methylprednisolone: 500 mg at day 0, 120 mg at day 1, then prednisone or equivalent,

6 20 mg/day from day 3. Maintenance immunosuppressive regimen was either based on

7 everolimus 0.75mg bid, targeted to 3-8 ng/ml and Cyclosporin A (CsA) with target ranges 100-

8 200 ng/ml from day 3 to month 2, 75-150 ng/mL from month 2 to month 4 and 25-50 ng/mL

9 from months 6 to 12, either Csa with target ranges of 150–220 ng/mL from day 3 to month 2,

10 100–150 ng/mL from month 2 to month 12 and mycophenolic acid 1080 mg bid for one month,

11 then 720 mg bid.

12

13 **Preparation of free CMV**

14 To produce free CMV (TB42/E strain), human foreskin fibroblasts were infected at a MOI of

15 0.1 and incubated at 37°C in culture medium DMEM, 8% bovine serum and glutamine for 10

16 days or until cytopathic effects were $\geq$90%. The supernatant was stored at -80°C. The

17 preparation had a titer of 2,5 10^6 PFU (plaque-forming unit)/ml, the titration was performed as

18 previously described [1]. All virus stocks and cells were tested negative for the presence of

19 *mycoplasma*.

20

21 **Viability of Vδ2^{neg} $\gamma\delta$ T cells during co-culture with and without blocking anti-CD3**

22 **antibody analyzed with DAPI**

23 Vδ2^{neg} $\gamma\delta$ T cells after 21 days of culture with 0 and 0.5 nM everolimus were negatively

24 sorted with magnetic beads and were cultured in medium alone (either with 0 or 0.5 nM

25   everolimus), non-infected (NI), CMV-infected fibroblasts with or without blocking anti-CD3

26   mAb (10µg/ml) during 24 hours and cells were stained with 1µM DAPI (4',6-Diamidino-2-

27   Phenylindole, Dihydrochloride) and after 15 minutes of incubation at room temperature,

28   cells were analyzed by flow cytometry Canto II.

29

Supplemental table 1. References of flow cytometry material

	Manufacturer	Clone	N°catalog	Last batch	dilution
perCP CD3	BD biosciences	SP34-2	9091596	552851	0.4
FITC CD16	BD biosciences	3g8	8255938	560996	
BV421 CD154	BD biosciences	TRAP-1	9171764	566268	0.1
Fixable viability stain 575v	BD biosciences		7020921	565694	0.0002
BV510 CD8	BD biosciences	SK-1	8003887	561617	0.1
BV650 CD137	BD biosciences	4B4-1	7194805	564092	0.1
BV786 CD27	BD biosciences	L128	8236716	563328	0.02
PE Pan-δ	Miltenyi	REA 591	5190321373	130-113-512	0.02
PC-7 Vδ2	Miltenyi	REA 711	5190131222	130-111-012	0.1
PE vio-615 CD103	Miltenyi	REA 803	5171115487	130-111-990	0.04
APC CD134	BD biosciences	ACT 35	7118719	563473	0.1
BV421 CD158a	BD biosciences	HP-3E4	7206680	564318	0.1
BV421 CD158b	BD biosciences	CH-L	8079900	743451	0.1
BV650 CD161	BD biosciences	DX12	7235832	563864	0.1
BV786 CD16	BD biosciences	3G8	7139586	563690	0.1
FITC CD85j	BD biosciences	GHI/75	6214793	555942	0.4
APC NKG2c	Miltenyi	REA 205	5190312085	130-117-547	0.04
PE vio-615 KLRG1	Miltenyi	REA 261	5171115662	130-108-395	0.06
BV650 PD1	BD biosciences	EH12	7258845	564104	0.1
BV711 TIM3	BD biosciences	7D3	8310921	565566	0.1
BV786 DNAM	BD biosciences	DX11	8318701	742497	0.04
APC CTLA4	BD biosciences	BNI3	7075573	555855	0.4
FITC granulysin	BD biosciences	RB1	7089964	558254	0.04
APC pan-δ	Miltenyi	REA591	5190314461	130-113-508	0.06
BV510 TRAIL	BD biosciences	NOK-1	7286506	744098	0.1
PE-CF594 CD8	BD biosciences	RPA-T8	7150677	562282	0.04
BV650 fas-ligand	BD biosciences	RIK-2	7284553	743721	0.1
V450 CD3	BD biosciences	UCHT1	8164556	560365	0.06
BV786 Interferon-γ	BD biosciences	4S-B3	7187947	563731	0.04
PE granzym B	Molecular probes	GB12	1735130	MHB04	0.04
APC-H7 CD45RA	BD biosciences	HI 100	7226562	560674	0,1
APC perforin	BD biosciences	dG9	7124573	563576	0,1
BV421 granzym	BD biosciences	GB11		563389	0,1
APC Cy7 CD16	BD biosciences	3G8	7075615	560195	0,1
FITC CD16	BD biosciences	3g8	8255938	560996	0.1
BV421 CD158a	BD biosciences	HP-3E4	9255329	564318	0,1
PC7 p-S6	Cell signalling	D57.2.2E	2	#34411	0,005
Alexa Fluor 647 p-38 MAPK	Cell signalling	3D7	7	#14594	0,02
PE pAkt T308	BD biosciences	J1-223.371	9192801	558275	0,1
FITC pERK 1/2	BD biosciences	20A	720836	612592	0,1
FITC pAkt S473	BD biosciences	M89-61	9282654	560404	0.1
PE Akt	BD biosciences	55/PKBa/Akt	8310873	560049	0,1
PE S6	Cell signalling	54D2	1	55594S	0,005
Alexa Fluor 647 Hobit (ZNF683)	BD biosciences	sanquin-hobit/1	8201604	566250	0.06
PC5.5 Tbet	ebioscience	P3.6.2.8.1	4289808	45-4714-80	0.06

PC7 Eomes	ebioscience	WD1928	192396	25-4877-42	0.06
PE Blimp1	BD biosciences	6D3	7290934	564702	0.02
foxp3 transcription factor staining buffer	fisher scientific		2075534	115-000-597	
BV510 CD3	BD biosciences	UCHT1	8297756	563109	0.06
live V780	BD biosciences		8207577	565388	0.0002
Pacific Blue TCR Vδ2	Beckman coulter	IMMU389	5	B49310	0.04
FITC TCR Vδ2	Beckman coulter	IMMU389	2000040	I1464	0.04
FITC KLRG1	ebioscience	13F13F2	4345831	53948842	0.4
PE CD69	Beckman coulter	TP1.55.3	41	IM19930	0.1
Alexa Fluor 700 CD69	BD biosciences	FN50	7258876	560739	0.1
PE-Cy5.5 panδ	Beckman coulter	IMMU510	30	A99021	01:50
live viability e fluor 780	ebioscience		4302692	65-0865-13	0.0002
BD Cytofix/cytoperm^{tm}	BD biosciences			555028	
Protein transport inhibitor	BD biosciences		9011506	554724	
phospho buffer perm III	BD biosciences		5260671	558050	
lyse/fix buffer 5X	BD biosciences		7180900	558049	
Purified anti-CD3	Beckman coulter	UCHT1	200036	IM1304	
Purified anti-Vδ1	produced in the lab				

31

32   **Supplemental Figures**

33   **Figure S1 Vδ2^{neg} γδ T cells and CD8+ αβ T cells phenotype at baseline in CMV**

34   **seropositive patients**

35   A. Vδ2^{neg} γδ T cell and CD8+ αβ T cell expression of CD27 and CD45RA analyzed by flow

36   cytometry separating patients with no CMV DNAemia or CMV DNAemia requiring no

37   treatment (well-controlled CMV, n=12) versus patients requiring CMV antiviral treatment

38   (severe CMV, n=9).

39   B. Vδ2^{neg} γδ T cells andCD8+ T cells phenotypes for inhibitory receptors and KLRG1

40   expression were validated in an internal cohort of patients with well-controlled CMV (n=5)

41   versus patients with severe CMV (n=7).

42   Each symbol represents an individual donor; large horizontal lines indicate the mean and small

43   horizontal lines indicate the standard deviation, 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001;

44   as determined by the Mann-Withney U test.

45

46

47   **Figure S2 Phenotype of Vδ2^{neg} γδ T cells and CMV-specific αβ T cells during in vitro**

48   **culture**

49   A. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro*

50   IL2-IL15 culture of Vδ2^{neg} γδ T cells, analyzed by flow cytometry.

51   B. One representative proliferation assay of TEMRA Vδ2^{neg} γδ T cells analyzed by CFSE

52   staining of CD45+ CD27- cells by flow cytometry.

53   C. One representative phenotype at day 7, 14 and 21 of CMV positive patient's PBMC during

54   *in vitro* culture of Vδ2^{neg} γδ T cells analyzed by flow cytometry.

55   D. Evolution of Vδ2^{neg} γδ T cell number during 21 days of PBMC cultures with IL2 (n=4) and

56   IL2-IL15 (n=4) ,obtained by Neubauer counting associated with analysis of Vδ2^{neg} γδ T cells

57   percentage by flow cytometry. Results are the mean $\pm$ SD of the cell number fold increase from

58   day 0, of PBMC cultures from those 4 different donors.

59   E. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro*

60   culture of CMV-specific $\alpha\beta$ T cells analyzed by flow cytometry.

61

62   **Figure S3 Low dose of mTORi decrease the number of Vδ2^{neg} $\gamma\delta$ T cells and CMV-specific**

63   **$\alpha\beta$ T cell expressing CD85j.**

64   Expression of CD85j was analyzed in Vδ2^{neg} $\gamma\delta$ T cells and CMV specific $\alpha\beta$ T cells by flow

65   cytometry after culturing PBMC of CMV seropositive donors (one representative, Figure A;

66   cumulative results for 5 donors, Figure B) *in vitro* with or without a low dose (0.5nM) of

67   everolimus during 21 days for the culture of Vδ2^{neg} $\gamma\delta$ T cells and during 16 days for the culture

68   of CMV specific $\alpha\beta$ T cells (stimulated overnight by pp65 peptides and gated on non-$\gamma\delta$ T cells

69   positive for CD69 and IFNγ). Each symbol represents an individual donor; $0.05 > p > 0.01$*;

70   **0.01 > p > 0.001$; as determined by Wilcoxon test.

71

72   **Figure S4 mTORi does not affect CMV-specific $\alpha\beta$ T cell frequencies during in vitro**

73   **culture**

74   One representative phenotype of CMV-specific $\alpha\beta$ T cells after 7 days of PBMC culture with

75   IL2 with or without 0.5nM everolimus. After overnight stimulation with CMV peptides, CMV-

76   specific $\alpha\beta$ T cells were gated by flow cytometry through their expression of CD69 and IFNg

77   among non $\gamma\delta$ T cells (left). Cumulative results for 5 donors(right).

78

79   **Figure S5 Blocking anti-CD3 mAb had no effect on Vδ2^{neg} $\gamma\delta$ T cell viability.**

80   Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM everolimus

81   and were cultured in the same medium alone (either with 0 or 0.5 nM everolimus), with non-

82   infected (NI), or with CMV-infected (CMV) fibroblasts with or without blocking anti-CD3

83   mAb (10µg/ml) during 24 hours and cells were stained with 1µM DAPI (4',6-Diamidino-2-

84   Phenylindole, Dihydrochloride) to assess their viability by flow cytometry.

85

86   **Figure S6 mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR**

87   **stimulation of Vδ2^{neg} γδ T cells**Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture

88   with 0 or 0.5 nM of everolimus , and stimulated with an agonist anti-CD3 mAb (UCHT1,

89   10µg/ml) for the indicated durations. SLP-76 phosphorylation (A) and MAP kinase 38

90   phosphorylation (B) were measured by flow cytometry in 4 donors (right, mean $\pm$ ranges) and

91   in a representative donor (left).

92

93

94   **Figure S7 No effect of mTORi on Akt phosphorylation after TCR stimulation of Vδ2^{neg}**

95   **γδ T cells**

96   After 21 days of culture with 0 or 0.5 nM of everolimus, PBMC were stimulated using an

97   agonist anti-Vδ1 mAb (10µg/ml) for 30 minutes, 2 and 4 hours and Akt phosphorylation was

98   measured and analyzed by flow cytometry (T308 phosphorylation site, left; S473

99   phosphorylation site, right) for 4 donors (mean $\pm$ ranges).

100

101   **References of supplemental methods**
102

103   1.   Matrosovich M, Matrosovich T, Garten W, Klenk HD. New low-viscosity overlay
104   medium for viral plaque assays. *Virol J*. 2006;3:63.
105

106

Figure S1
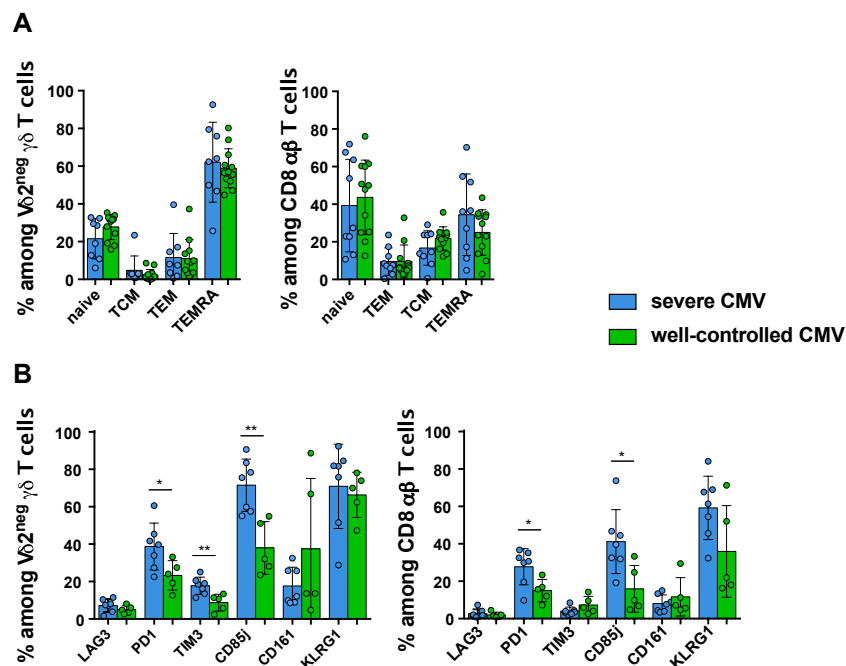
Figure S1 Vδ2ⁿᵉᵍ γδ T cells and CD8+ αβ T cells phenotype at baseline in CMV seropositive patients

A. Vd2ⁿᵉᵍ γδ T cell and CD8+ αβ T cell expression of CD27 and CD45RA analyzed by flow cytometry separating patients with no CMV DNAemia or CMV DNAemia requiring no treatment (well-controlled CMV, n=12) versus patients requiring CMV antiviral treatment (severe CMV, n=9).

B. Vδ2ⁿᵉᵍ γδ T cells and CD8+ T cells phenotypes for inhibitory receptors and KLRG1 expression were validated in an internal cohort of patients with well-controlled CMV (n=5) versus patients with severe CMV (n=7).

Each symbol represents an individual donor; large horizontal lines indicate the mean and small horizontal lines indicate the standard deviation, 0.05>p>0.01*; **0.01>p>0.001; ***p<0.001; as determined by the Mann-Withney U test.

Figure S2
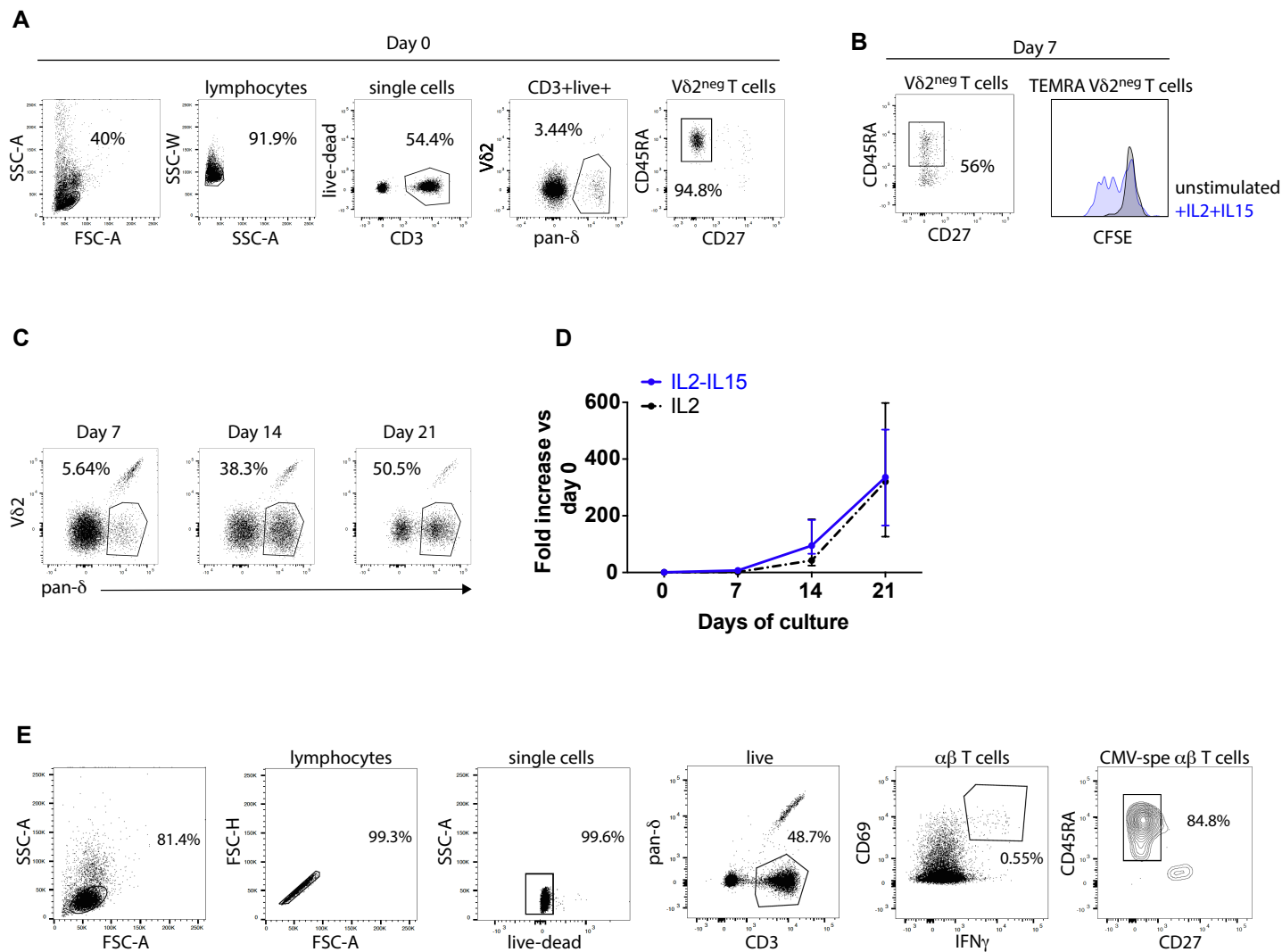
Figure S2 Phenotype of Vδ2^neg γδ T cells and CMV-specific αβ T cells during in vitro culture

A. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* IL2-IL15 culture of Vδ2^neg γδ T cells, analyzed by flow cytometry. B. One representative proliferation assay of TEMRA Vδ2^neg γδ T cells analyzed by CFSE staining of CD45+ CD27- cells by flow cytometry. C. One representative phenotype at day 7, 14 and 21 of CMV positive patient's PBMC during *in vitro* culture of Vδ2^neg γδ T cells analyzed by flow cytometry. D. Evolution of Vδ2^neg γδ T cell number during 21 days of PBMC cultures with IL2 (n=4) and IL2-IL15 (n=4) ,obtained by Neubauer counting associated with analysis of Vδ2^neg γδ T cells percentage by flow cytometry. Results are the mean ± SD of the cell number fold increase from day 0, of PBMC cultures from those 4 different donors. E. One representative phenotype at day 0 of CMV positive patient's PBMC used for *in vitro* culture of CMV-specific aβ T cells analyzed by flow cytometry.

A
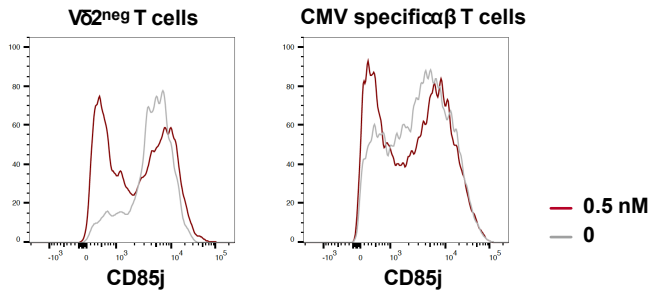
B
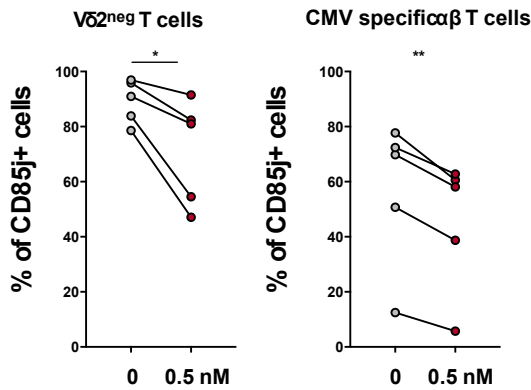
Figure S3 Low dose of mTORi decrease the number of Vδ2^{neg} γδ T cells and CMV-specific αβ T cell expressing CD85j.

Expression of CD85j was analyzed in Vδ2^{neg} γδ T cells and CMV specific αβ T cells by flow cytometry after culturing PBMC of CMV seropositive donors (one representative, Figure A; cumulative results for 5 donors, Figure B) *in vitro* with or without a low dose (0.5nM) of everolimus during 21 days for the culture of Vδ2^{neg} γδ T cells and during 16 days for the culture of CMV specific αβ T cells (stimulated overnight by pp65 peptides and gated on non-gδ T cells positive for CD69 and IFNγ). Each symbol represents an individual donor; $0.05 > p > 0.01$*; **$0.01 > p > 0.001$; as determined by Wilcoxon test.
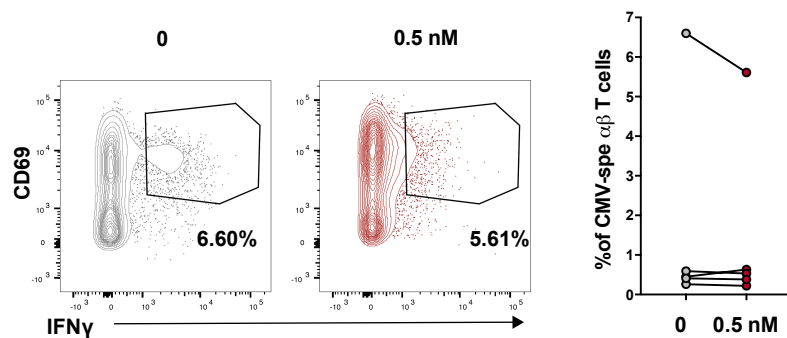
Figure S4 mTORi does not affect CMV-specific αβ T cell frequencies during in vitro culture

One representative phenotype of CMV-specific aβ T cells after 7 days of PBMC culture with IL2 with or without 0.5nM everolimus. After overnight stimulation with CMV peptides, CMV-specific αβ T cells were gated by flow cytometry through their expression of CD69 and IFNγ among non γδ T cells (left). Cumulative results for 5 donors(right).
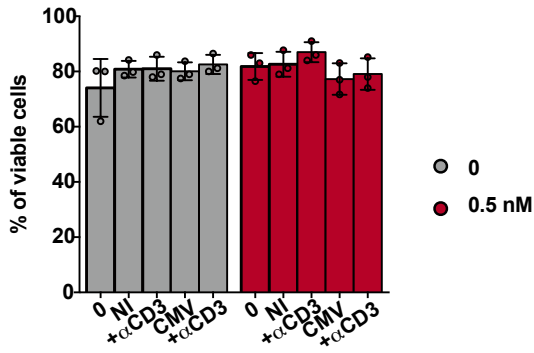
Figure S5 Blocking anti-CD3 mAb had no effect on Vδ2^{neg} γδ T cell viability.

Vδ2^{neg} γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM everolimus and were cultured in the same medium alone (either with 0 or 0.5 nM everolimus), with non-infected (NI), or with CMV-infected (CMV) fibroblasts with or without blocking anti-CD3 mAb (10μg/ml) during 24 hours and cells were stained with 1μM DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) to assess their viability by flow cytometry.

A

B
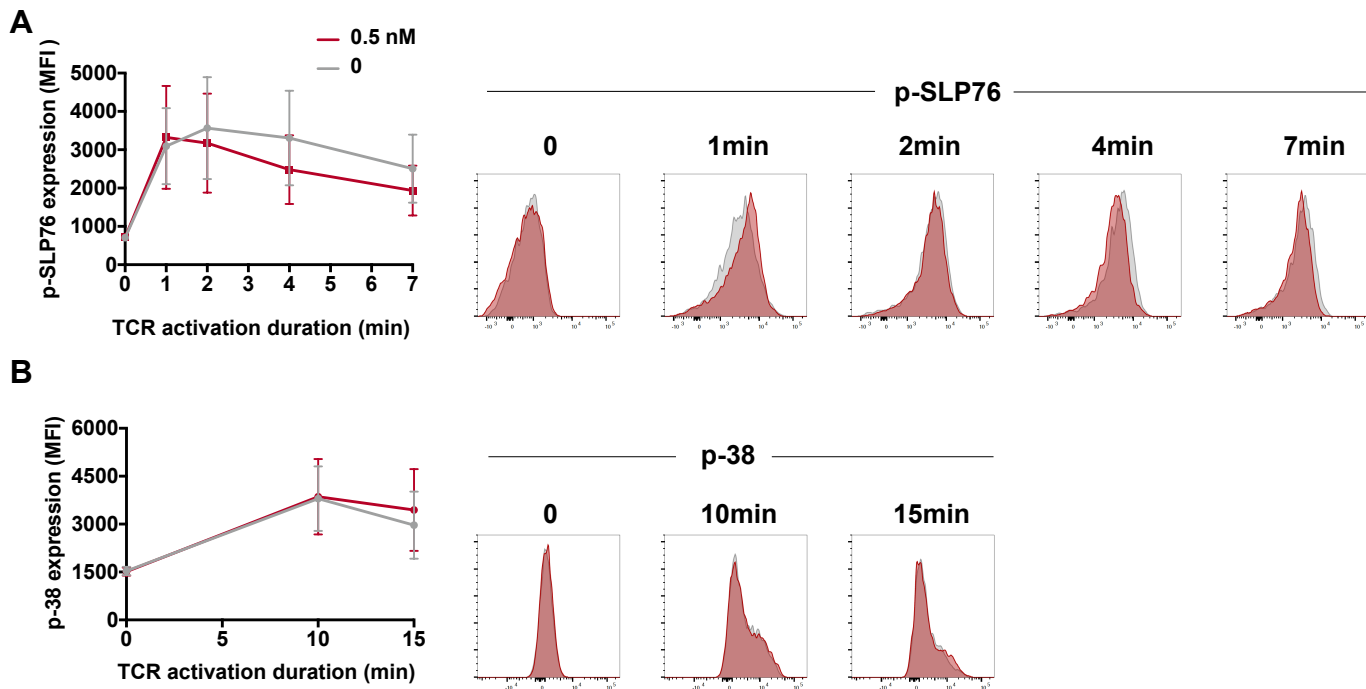
Figure S6 mTORi does not affect SLP-76 and MAP kinase 38 signaling after TCR stimulation of Vδ2[neg] γδ T cells

Vδ2[neg] γδ T cells were negatively sorted after 21 days of culture with 0 or 0.5 nM of everolimus , and stimulated with an agonist anti-CD3 mAb (UCHT1, 10μg/ml) for the indicated durations. SLP-76 phosphorylation (A) and MAP kinase 38 phosphorylation (B) were measured by flow cytometry in 4 donors (right, mean ± ranges) and in a representative donor (left).
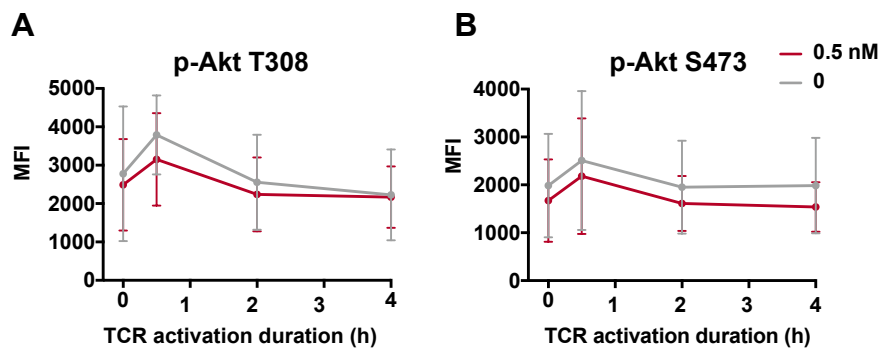
A

p-Akt T308

B

p-Akt S473

— 0.5 nM
— 0

Figure S7 No effect of mTORi on Akt phosphorylation after TCR stimulation of Vδ2^{neg} $\gamma\delta$ T cells

After 21 days of culture with 0 or 0.5 nM of everolimus, PBMC were stimulated using an agonist anti-Vδ1 mAb (10μg/ml) for 30 minutes, 2 and 4 hours and Akt phosphorylation was measured and analyzed by flow cytometry (T308 phosphorylation site, left; S473 phosphorylation site, right) for 4 donors (mean $\pm$ ranges).
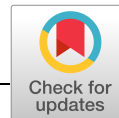
ARTICLE 6

ORIGINAL ARTICLE

Microbiology and Immunology WILEY

Effect of mTOR inhibitors during CMV disease in kidney transplant recipients: Results of a pilot retrospective study

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Abstract

mTOR inhibitors exert a preventive effect on cytomegalovirus (CMV) disease in CMV seropositive (R+) kidney transplant recipients, but their impact during the curative treatment of CMV disease in high-risk kidney transplant recipients has not been investigated. We aimed to evaluate the efficacy and tolerance of mTOR inhibitors compared with mycophenolic acid in 63 consecutive kidney transplant recipients (80% of D+R−) suffering from CMV disease with a persistent or a recurrent CMV DNAemia. In this monocentric retrospective study, 16 had their treatment converted to mTOR inhibitors and 47 did not. The Kaplan–Meier curves did not show any significant differences in CMV DNAemia eradication (77% vs. 88% respectively; hazard ratio (HR), 1.648 [95% confidence interval (CI), 0.913–2.973]; log-rank test, $P = .132$), DNAemia recurrence (36% vs. 47%; HR, 1.517 [95% CI, 0.574–4.007]; log-rank test, $P = .448$) and CMV clinical recurrence (17% vs. 27%; HR, 1.375 [95% CI, 0.340–5.552]; log-rank test, $P = .677$) between patients who received mTOR inhibitors and those who did not. These results were confirmed in uni- and multivariate time-dependent Cox regressions. In summary, conversion from mycophenolic acid to mTOR inhibitors seems inadequate for improving CMV clearance or in better preventing CMV recurrences during severe or persistent CMV disease.

KEYWORDS

CMV persistence, CMV recurrence, cytomegalovirus, kidney transplantation, mTOR inhibitors

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1 | INTRODUCTION

Despite the current prevention strategies, cytomegalovirus (CMV) is still a major issue in solid-organ transplant recipients. CMV disease is very frequent with an incidence reaching 15–20% in D+R−[1–3] and 5% in R+ kidney transplant recipients (KTR).[4] Moreover, despite appropriate antiviral treatment during CMV disease, failure to clear CMV occurs in 30% of patients. Finally, DNAemia and clinical recurrences still occur in 30% and 15% of KTR, respectively.[5] Therefore, there is an unmet need for new strategies to better treat CMV disease and to avoid recurrence.

Over the past years, a growing body of evidence, including randomized controlled trials, has shown that the use of mTOR inhibitors (mTORi) had anti-CMV properties. In vitro, mTORi could directly inhibit CMV replication, potentiate the generation of CMV-specific memory CD8+ T-cells, and deviate the CMV-mediated immune evasion by blocking mTORC1 activity in myeloid cells.[6,7] In randomized trials, a reduction of post-transplant CMV events was reported in kidney transplant recipients receiving de novo mTORi, either in association with mycophenolic acid (MPA),[8] or with calcineurin inhibitors (CNI).[9,10,11] Based on these studies, CMV guidelines now propose the use of mTORi as a potential preventive approach to decrease CMV infection and disease in R+ kidney transplant recipients.[12]

While mTORi are able to exert a preventive effect on CMV disease in R+ kidney transplant recipients, their use during CMV disease to achieve faster CMV eradication has not been demonstrated. Two analyses have been made in 9 and 11 patients and it was reported that switching immunosuppression from CNI to a mTORi-based regimen could be proposed as a salvage therapy in ganciclovir-resistant CMV infection.[13,14] However, the use of mTORi in the clinical scenarios of CMV DNAemia persistence or recurrence has not been investigated, notably switching from MPA to mTORi with calcineurin inhibitors maintenance. Indeed, as mycophenolate mofetil (the precursor of MPA) has been shown to increase CMV invasive organ diseases in kidney transplant recipients,[15] changing to mTORi could offer an option against the deleterious effect of MPA.

We therefore conducted a retrospective study to evaluate the efficacy and tolerance of a conversion from MPA to mTORi in kidney transplant recipients suffering from CMV disease with a persistent or a recurrent CMV DNAemia.

2 | PATIENTS AND METHODS

2.1 | Study design

A monocentric retrospective study was conducted at the Bordeaux University Hospital, France, from April 2009 to April 2018. Among the consecutive 95 patients who underwent CMV disease during this period, first 20 patients were excluded with early CMV eradication without recurrence. Also excluded were the patients for whom mTORi had been already administered in other indications ($n = 10$), as well as multiple organ transplantations ($n = 2$). Consequently, 63 patients were included, either patients who had a persistent CMV DNAemia after 7 weeks of antiviral treatment ($n = 22$), based on the VICTOR study[5] or those who had a CMV recurrence ($n = 41$; Figure 1). All patients were monitored for 1 year after meeting the inclusion criteria. This study was approved by the Institutional Review Board of the Bordeaux University Hospital.

2.2 | Immunosuppression

Induction therapy was based on rabbit anti-thymocyte globulins (rATG) in sensitized patients, and basiliximab in non-sensitized patients. The immunosuppressive regimen was based on CNI with a tacrolimus target residual plasma concentration of 10–12 ng/mL for the first 3 months and then 5–10 ng/mL. The cyclosporine A target residual plasma concentration was 150–200 ng/mL for the first 3 months and then 75–125 ng/mL. MPA was used mainly as an antiproliferative drug until the clinical decision was made to switch to mTORi for the indication of CMV persistence or CMV recurrence during an outpatient visit. No standardized criteria were used to define patients eligible for mTORi conversion. Conversion from MPA to mTORi was decided clinically because mTORi has been demonstrated to prevent CMV infection better than MPA, to contribute to CMV-mediated evasion suppression,[6] and consequently has been recently added in the latest CMV guidelines.[12]

We considered the beneficial effect of mTORi to outweigh its adverse events such aphtosis, edemas, and delay in healing. Those adverse events are described below. The mTORi target residual plasma concentration was then 4–7 ng/mL. All patients received everolimus as mTORi treatment, except one who received rapamycin.

The expanded criteria for donor and delayed graft function were defined as described previously.[16] All acute rejections, which included both antibody-mediated and T cell-mediated acute rejections, were biopsy-proven. The glomerular filtration rate was expressed based on the MDRD formula.

2.3 | CMV prevention

From April 2009 to June 2010, patients were preemptively followed and treated when the CMV
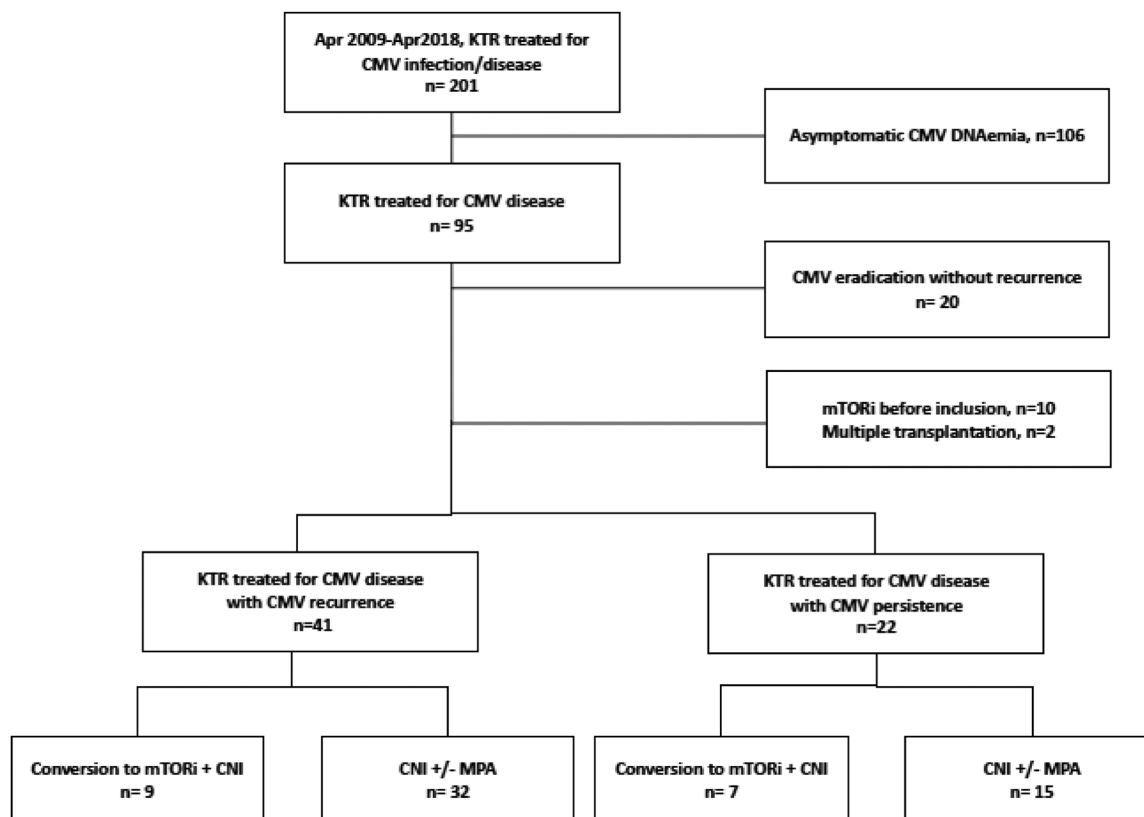
FIGURE 1 Flow chart of study design. Repartition of patients are described. Between April 2009 and April 2018, 201 kidney transplant recipients (KTR) underwent CMV infection. Patients with asymptomatic CMV viremia were excluded (*n* = 106). Among 95 KTR with CMV disease, 20 were excluded because they had CMV eradication and no recurrence, 10 were excluded because were already treated with an mTOR inhibitor before inclusion for other reasons and 2 because of multiple transplantation. Twenty-two patients had CMV persistence of which 7 had mTORi conversion. Forty-one patients had CMV recurrence of which nine had mTORi conversion. Apr, April; CMV, cytomegalovirus; KTR, kidney transplant recipient; mTORi, mTOR inhibitor; n, number

quantitative nucleic acid testing (QNAT) was 2000 IU/mL as reported previously.[17] From July 2010 to April 2018, D+R− and R+ patients received, respectively, 6 months and 3 months of universal prophylaxis using valganciclovir 900 mg once a day. The dose was carefully adjusted at each outpatient visit, following the manufacturer's recommendations, using the Cockcroft–Gault formula.

2.4 | CMV monitoring

The CMV IgG serology (Enzygnost anti-CMV/IgM and IgG [Dade Behring, Marburg, Germany] and Acces CMV IgG and IgM [Beckman Coulter, Brea, CA]), were performed following the manufacturer's recommendations.

CMV QNAT was performed as described previously with a real-time polymerase chain reaction in whole blood,[18] and from June 2012, using LightMix® kit human cytomegalovirus (hCMV; TIB MOLBIOL GmbH, Berlin, Germany). Before June 2012, QNAT was reported in copies/mL, however, the equivalence in IU/mL was

retrospectively calculated by using the World Health Organization International Standard for hCMV, in order to homogenize all the results before performing the statistical analysis. The threshold of CMV DNAemia detectability was 250 IU/mL. The laboratory is following the program of Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) from 2004.

The baseline viral load was defined as the first positive DNAemia of the CMV event which led us to include the patient. During the viral monitoring of CMV disease, the assay was performed once a week during the first 7 weeks or until two consecutive negative CMV QNATs had occurred, then once a month until month 3. CMV QNAT was also systematically performed every year and when CMV disease was clinically suspected.

Antiviral drug resistance was suspected when persistent viral replication was observed after >2 weeks of appropriate antiviral therapy and was confirmed by full-length sequencing of the UL97 and UL54 genes,[3] performed at the French National Cytomegalovirus Reference Center (Limoges, France).

2.5 | Definitions of CMV events

CMV disease was defined as CMV syndrome or CMV tissue-invasive disease, consistent with the American Society of Transplantation and the CMV Drug Development Forum recommendations.[19] Intravenous (i.v.) ganciclovir (5 mg/kg twice daily) or oral valganciclovir (900 mg twice daily) were given as the curative treatment. The dose was carefully adjusted at each outpatient visit, following the manufacturer's recommendations, using the Cockcroft–Gault formula.

"Post-prophylaxis" CMV disease was defined as the first episode of CMV disease occurring >3 months (100 days) after transplantation.

Early-onset disease was defined as the first episode of CMV disease occurring <3 months (100 days) after transplantation.[20]

CMV DNAemia eradication was defined as the occurrence of one negative CMV QNAT.

CMV DNAemia persistence was defined as no CMV eradication occurring after 7 weeks of antiviral treatment.

CMV DNAemia recurrence was assessed as a new positive CMV DNAemia (≥ 250 IU/mL) in patients with proven CMV DNAemia eradication. Clinical recurrence was defined as CMV DNAemia recurrence associated with viral syndrome or documentation of CMV in tissue from a relevant organ.[12]

2.6 | Immunophenotyping

We have previously shown that $V\delta 2^{neg}$ T lymphocytes are involved in the control of CMV[21] since their expansion is correlated with CMV infection resolution.[22] Consequently, $V\delta 2^{neg}$ T lymphocytes kinetics were analyzed in patients with or without conversion to mTORi. The $V\delta 2^{neg}$ T lymphocyte count was obtained by immunophenotypic determination. Flow cytometry was carried out on 100 µL anticoagulated whole blood taking into account at least 5000 total lymphocytes stained with anti-CD45, anti-pan-δ (clone IMMU 510; Beckman Coulter, Krefeld, Germany), and anti-TCR Vδ2 (clone 15D; Thermo Fisher Scientific, Rockford, IL). The percentages of cell populations were obtained using CELL-QUEST software (BD Bioscience), and absolute counts of lymphocytes were obtained using the Single–Platform Lyse/No–Wash Trucount (BD Bioscience). In our center, the surveillance of $V\delta 2^{neg}$ T lymphocytes was based on a measurement at day 0 of the graft; month 3, 6, and 12; and then every year. In the case of CMV persistence, the monitoring was performed at day 0 of CMV infection, day 49 and once a month in the 3 months following day 49. In the case of CMV recurrence, the surveillance of $V\delta 2^{neg}$ T lymphocytes was performed on that day and then once a month in the three following months.

2.7 | Statistical analysis

The Mann-Whitney test and the χ^2 test were used when appropriate. Alternatively to the χ^2 test, the Fisher's test was used for a low number of patients. $P < .05$ was considered statistically significant. The survival curves were estimated with Kaplan–Meier method and compared with the log-rank test.

We performed a univariate time-dependent Cox regression analysis for CMV DNAemia eradication, CMV DNAemia recurrence and clinical recurrence. Then, covariates with $a < 0.25$ P-value and "mTORi conversion" were included in a multivariate time-dependent Cox regression analysis. The results were expressed as hazard ratios (HR) with 95% confidence intervals (95% CI). Finally, we separately analyzed patients with persistent and recurrent CMV disease.

Analyses were performed with conventional statistical methods using the GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA) and the RStudio statistical software (Version 1.1.423 – © 2009-2018 RStudio, Inc).

3 | RESULTS

3.1 | Baseline characteristics of patients

Sixty-three KTR with CMV disease were included at the time of persistent CMV DNAemia diagnosis or at the time of CMV DNAemia recurrence. Baseline characteristics are described in Table 1. Seventy percent of the patients were males with an average age of 56 years. Forty-one percent received rATG as induction treatment, 79.4% received tacrolimus, while 17.5% received cyclosporine A. Seventy percent had corticosteroids and 92% had MPA. Acute antibody-mediated rejection occurred before inclusion in 1.6% of patients and acute T cell-mediated rejection in 11% of patients.

Among the 63 included patients, 16 were converted to mTORi (Table 2). The conversion to mTORi occurred 31 days after inclusion on average (median 21; 1st–3rd quartile 8–49 days). Among the 16 patients converted to mTORi, 56% still received ganciclovir or valganciclovir at the time of the conversion, versus 45% in the patients without mTORi conversion ($P = .675$). In the other patients, antiviral therapy was stopped.

The proportions of CMV diseases with persistent CMV DNAemia, D+/R− status, and of a mutant strain

TABLE 1 Baseline characteristics of patients at inclusion

Characteristic	Value
Gender	
Female/Male	19/63 (30%)/44/63 (70%)
Age (yr), mean ± SD	56 ± 13
Rank of transplantation	
1	53/63 (84%)
>1	10/63 (15.8%)
Preemptive transplantation	
No/Yes	7/63 (11%)/56/63 (89%)
Total ischemia time (hours), mean ± SD	15 ± 7.7
Delayed graft function	
No/Yes	39/63 (62%)/24/63 (38%)
Expanded criteria donor	
No/Yes	23/63 (37%)/34/63 (54%)
NA	6/63 (9%)
Living donor	
No/Yes	56/63 (89%)/7/63 (11%)
Nephropathy	
Vascular	18/63 (29%)
Glomerular	17/63 (27%)
Unknown	8/63 (13%)
Tubulo interstitial/others	12/63 (19%)
Hereditary/others	8/63 (13%)
Immunosuppressive regimen	
rATG induction	
No/Yes	36/61 (59%)/25/61 (41%)
Cyclosporine A	
No/Yes	52/63 (82.5%)/11/63 (17.5%)
Tacrolimus	
No/Yes	13/63 (20.6%)/50/63 (79.4%)
MPA	
No/Yes	5/63 (8%)/58/63 (92%)
Corticosteroids	
No/Yes	19/63 (30%)/44/63 (70%)
Azathioprine	
No/Yes	60/63 (95.2%)/3/63 (4.7%)
Previous ABMR	
No/Yes	62/63 (98%)/1/63 (1.6%)
Previous TCMR	
No/Yes	56/63 (89%)/7/63 (11%)

This table describes the baseline characteristics of patients at inclusion, regarding age, sex, rank of transplantation, graft characteristics, immunosuppressive regimen, and the history of acute rejection before inclusion.

Abbreviations: ABMR, antibody-mediated rejection; CMV, cytomegalovirus; rATG, rabbit anti-thymocyte globulins; TCMR, T cell-mediated rejection; SD, standard deviation.

were not significantly different in the two groups (Table 2). The mean of the absolute lymphocytes counts was slightly higher in the group without mTORi conversion (635 vs. 785/μL, respectively), but it was not statistically significant ($P = .058$). The preventive strategy, the baseline viral load, the ratio of "post-prophylaxis" versus "early-onset disease" between the two groups were not significantly different either (Table 2).

3.2 | mTOR inhibitors conversion is not associated with a faster CMV DNAemia eradication

Univariate analysis showed no significant association between mTORi conversion and CMV DNAemia eradication (HR, 0.835; [95% CI, 0.411–1.696]; $P = .618$; Table 3a). Analysis of the 1-year survival curve showed no significant difference of CMV DNAemia eradication between patients with or without mTORi conversion (77% vs. 88% respectively; HR, 1.648 [95% CI, 0.913–2.973]; log-rank test, $p = .132$; Figure 2a).

In the multivariate analysis, only the "persistent CMV DNAemia versus recurrent CMV infection" covariate was associated with a lower rate of CMV DNAemia eradication (HR, 0.438 [95% CI, 0.221–0.718]; $P = .002$; Table 3A).

3.3 | mTOR inhibitors is not associated with CMV recurrence

We then excluded the patients for whom CMV DNAemia eradication was not obtained ($n = 11$, four with mTORi conversion and seven without mTORi conversion), in order to analyze the covariates associated with both CMV DNAemia and clinical recurrences.

mTORi conversion was not significantly associated with either CMV DNAemia recurrence (HR, 0.942; [95% CI, 0.314–2.831]; $P = .916$; Table 3b), or clinical recurrence (HR, 1.524; [95% CI, 0.408–5.693]; $P = .531$; Table 3c). The CMV DNAemia recurrence (36% vs. 47%; HR 1.517 [95% CI, 0.574–4.007]; log-rank test, $P = .448$; Figure 2b) and the clinical recurrence (17% vs. 27%; HR, 1.375 [95% CI, 0.340–5.552]; log-rank test, $P = .677$; Figure 2c) were not significantly different in patients with and without mTORi conversion in the 1-year survival curves.

In the multivariate analyses, only rATG was significantly associated with an increased risk of both DNAemia (HR, 3.581; [95% CI, 1.449–8.848]; $P = .005$; Table 3b) and clinical recurrence (HR, 7.723; [95% CI, 2.042–29.2]; $P = .002$; Table 3c). The percentage of patients receiving rATG was not different in the patients

TABLE 2 Baseline characteristics relative to CMV disease

Characteristic	Conversion to mTORi, $n = 16$	No conversion to mTORi, $n = 47$	P-value
Recurrent/persistent	9/16 (56%)/7/16 (44%)	32/47 (68%)/15/47 (32%)	.391
CMV status D+R-/R+	13/16 (81%)/3/16 (19%)	36/47 (77%)/11/47 (23%)	.698
Preemptive versus universal prophylaxis	2/16 (12%)/14/16 (88%)	13/47 (28%)/34/47 (72%)	.315
Early-onset versus "post-prophylaxis" CMV disease	2/16 (12%)/14/16 (88%)	11/47 (23%)/36/47 (77%)	.486
No/VGV/GCV/FCV at inclusion			.675
	6/16 (37.5%)	26/47 (55%)	
	7/16 (43.75%)	15/47 (32%)	
	2/16 (12.5%)	6/47 (13%)	
	1/16 (6.25%)	0	
Baseline CMV DNAemia (IU/ml); mean ± SD	685720 ± 1447761	753024 ± 1924860	.869
Mutant strain	14/16 (88%)/2/16 (12%)	43/47 (91%)/4/47 (8.5%)	
Lymphocyte count (/μL); mean ± SD	635 ± 647	785 ± 464	.058
rATG	7 (43.8)	18 (38.4)	.7

This table describes CMV baseline characteristics of the donor and recipient status, type of preventive strategy, inclusion for CMV persistence or recurrence, the antiviral treatment, virus characteristics, and lymphocyte count.
Abbreviations: CMV, cytomegalovirus; mTORi, mTOR inhibitors; rATG, rabbit anti-thymocyte globulins; SD, standard deviation.

TABLE 3a Uni- and multivariate analysis for CMV DNAemia eradication

Univariate analysis				Multivariate analysis		
Covariate	HR	CI 95%	P-value	HR	CI 95%	P-value
Age	1.001	0.981–1.021	0.949			
Sex	0.972	0.530–1.784	.928			
rATG	0.861	0.479–1.549	.619			
Corticosteroids	1.195	0.651–2.19	.566			
mTORi conversion	0.835	0.411–1.696	.618			
CMV status	1.194	0.609–2.338	.606			
Preventive strategy	0.423	0.225–0.793	.007			
Early-onset disease	0.429	0.255–0.95	.034			
Mutant strain	0.540	0.194–1.504	.239			
Baseline viral load	0.473	0.269–0.831	.009			
Persistent versus recurrent	0.376	0.204–0.693	.001	0.438	0.221–0.718	.002
TCMR before CMV disease	1.526	0.644–3.609	.336			
ABMR before CMV disease	3.011	0.401–21.58	.284			
Lymphocyte count	1	1–1.001	.074	1.0005	1–1.0011	.068

Baseline viral load was converted in a binary variable, taking the median value of 58,000 IU/mL and analyzing the correlation between baseline viral load higher or lower than this cutoff and CMV DNAemia eradication. Seven variables were analyzed kept for the initial multivariate analysis and the model was convergent.
Abbreviations: ABMR, antibody-mediated rejection; CI, confidence interval; CMV, cytomegalovirus; HR, hazard ratio; mTORi, mTOR inhibitors; rATG, rabbit anti-thymocyte globulins; TCMR, T cell-mediated rejection.
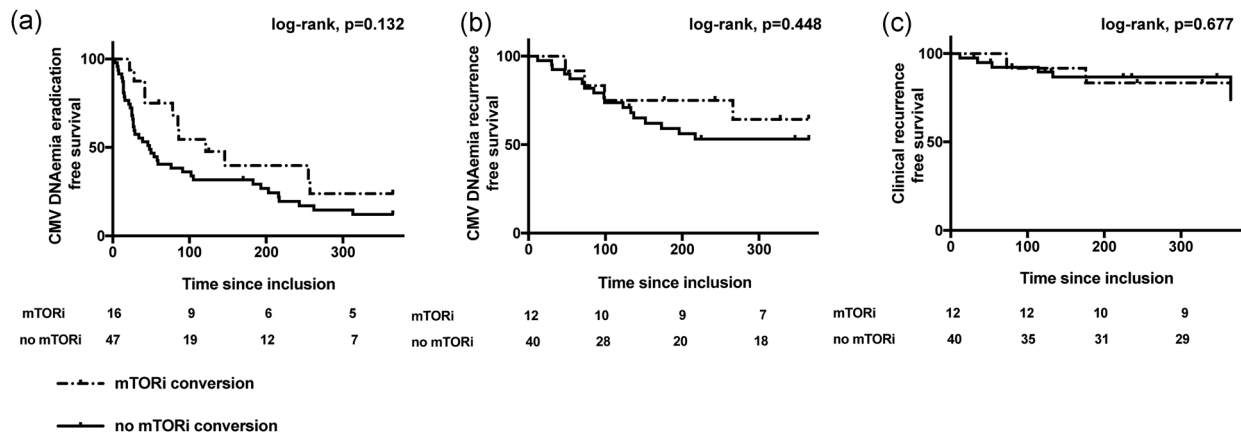
FIGURE 2 Kaplan–Meier curves comparison between patients with or without mTOR inhibitor conversion for CMV outcomes. (a) CMV DNAe. Kaplan–Meier curves for CMV DNAemia eradication free-survival in patients with and without mTORi conversion. Patients with and without mTORi conversion did not have a significant difference in CMV DNAemia eradication (77% vs. 88% respectively; HR, 1.648 [95% CI, 0.913–2.973]; log-rank test, $P = .132$). (b) CMV DNAemia recurrence. Kaplan-Meier curves for CMV DNAemia recurrence free-survival in patients with and without mTORi conversion. Patients with and without mTORi conversion did not have a significant difference in the CMV DNAemia recurrence occurrence (36% vs. 47%; HR 1.517 [95% CI, 0.574–4.007]; log-rank test, $P = .448$). (c) CMV clinical recurrence. Kaplan-Meier curves for CMV recurrence free-survival in patients with and without mTORi conversion. Patients with and without mTORi conversion did not have a significant difference in the clinical recurrence occurrence (17% vs. 27%; HR, 1.375 [95% CI, 0.340–5.552]; log-rank test, $P = .677$). CI, confidence interval; CMV, cytomegalovirus; HR, hazard ratio; mTORi, mTOR inhibitor

with and without mTORi conversion (43.8% vs. 38.4% respectively, $P = .7$).

3.4 | Analysis of the anti-CMV immune response using the Vδ2^{neg} T lymphocyte expansion

Baseline characteristics were similar in patients with or without mTORi conversion in the two subgroups: "CMV persistence" and "CMV recurrence", with the exception of lymphocyte counts which were lower in patients converted to mTORi in the CMV persistent subgroup (326 vs. 430/μL, respectively, $P = .01$; Table 4a, b).

One-year CMV DNAemia eradication and CMV DNAemia recurrence were also similar in patients with or without mTORi conversion in these two subgroups (subgroup CMV persistence; $P = .823$ and .387 respectively. Subgroup CMV recurrence; $P = .061$ and .661 respectively, Table 4a, b).

Finally, CMV-specific Vδ2^{neg} T lymphocyte expansion were also similar in patients with or without mTORi conversion in these two subgroups (Figure 3a, b)

3.5 | Rejection and graft survival

Since tacrolimus was mainly used, we compared the tacrolimus trough levels between the two groups at 1-, 3-,

6-, and 12-months post-inclusion, and observed that the tacrolimus trough level at month 3 was significantly lower in patients with mTORi conversion (Figure S1). Patients converted to mTORi exhibited few adverse events, as described in Table S1. Finally, acute rejection, eGFR, graft, and patient survival were compared 1-year post-inclusion and no significant difference was noted between the two groups (Table S2).

4 | DISCUSSION

It is now widely accepted that the use of mTORi is a potential approach to decrease the incidence of CMV disease in R+ kidney transplant recipients. On the contrary, this retrospective study shows that the conversion from MPA to mTORi seems inadequate in improving CMV clearance or in better preventing CMV recurrences of persistent and recurrent CMV disease. Hypotheses can be proposed to explain this lack of efficacy in those scenarios.

First of all, this finding was observed in a very carefully selected population, displaying the most difficult CMV diseases to treat. For patients with persistent CMV DNAemia after 7 weeks of antiviral treatment, the management based on international recommendations remains controversial. Treating patients until obtaining a CMV QNAT below the lower limit of quantification on one highly sensitive assay, or two consecutive negative results if the assay is not highly sensitive is

TABLE 3b Uni and multivariate analysis for CMV DNAemia recurrence

Univariate analysis				Multivariate analysis		
Covariate	HR	CI 95%	P-value	HR	CI 95%	P-value
Age	1.006	0.974–1.038	.726			
Sex	0.535	0.223–1.282	.161			
rATG	3.956	1.625–9.63	.002	3.581	1.449–8.848	.005
Corticosteroids	1.402	0.546–3.595	.482			
mTORi conversion	0.942	0.314–2.831	.916			
CMV status	1.687	0.655–4.344	.278			
Preventive strategy	0.682	0.277–1.675	.404			
Early-onset disease	0.571	0.232–1.402	.221			
Mutant strain	2.093	0.618–7.087	.235			
Baseline viral load	1.197	0.518–2.762	.674			
Persistent versus recurrent	0.569	0.209–1.547	.27			
TCMR before CMV disease	0.295	0.039–2.196	.233			
ABMR before CMV disease	1.086	0.988–1.122	.998			
Lymphocyte count	0.680	0.294–1.572	0.368			

Baseline viral load was converted in a binary variable, taking the median value of 58,000 IU/mL and analyzing the correlation between baseline viral load higher or lower than this cutoff and CMV DNAemia eradication. Five variables were kept for the initial multivariate analysis and the model was convergent. Abbreviations: ABMR, antibody-mediated rejection; CI, confidence interval; CMV, cytomegalovirus; HR, hazard ratio; mTORi, mTOR inhibitors; rATG, rabbit anti-thymocyte globulins; TCMR, T cell-mediated rejection.

TABLE 3c Uni- and multivariate analysis for clinical recurrence

Univariate analysis				Multivariate analysis		
Covariate	HR	CI 95%	P-value	HR	CI 95%	P-value
Age	1.013	0.968–1.06	.578			
Sex	0.290	0.092–0.911	.0341			
rATG	6.386	1.718–23.73	.005	7.723	2.0422–29.2	.002
Tacrolimus	1.233	0.333–4.558	.754			
Corticosteroids	2.559	0.559–11.71	.226			
mTORi conversion	1.524	0.408–5.692	.531			
CMV status	2.256	0.677–7.516	.185			
Preventive strategy	5.07	0.652–39.37	.121			
Early-onset disease	4.317	0.555–33.54	.162	5.806	0.7313–46.1	.096
Mutant strain	3.014	0.656–13.84	.156			
Baseline viral load	1.294	0.417–4.012	.656			
Persistent versus recurrent	0.894	0.241–3.313	.867			
TCMR before CMV disease	1.184e-08	0-Inf	.998			
ABMR before CMV disease	2.959e-07	0-Inf	.998			
Lymphocyte count	0.323	0.097–1.077	.066			

Baseline viral load was converted in a binary variable, taking the median value of 58,000 IU/mL and analyzing the correlation between baseline viral load higher or lower than this cutoff and CMV DNAemia eradication. We put the seven most significantly associated variables to obtain a convergent model. Abbreviations: ABMR, antibody-mediated rejection; CI, confidence interval; CMV, cytomegalovirus; HR, hazard ratio; mTORi, mTOR inhibitors; rATG, rabbit anti-thymocyte globulins; TCMR, T cell-mediated rejection.

TABLE 4a Baseline characteristics in "CMV persistent" group

Characteristics	Conversion to mTORi, $n = 7$	No conversion to mTORi, $n = 15$	P-value
CMV status D+R−/R+	1 (14.3)/6(85.7)	12 [80]/3 (20)	.746
Preemptive versus universal prophylaxis	1 (14.3)/6(85.7)	0/15	.318
Early-onset versus "post-prophylaxis" CMV disease	1 (14.3)/6(85.7)	0/15	.318
No/VGV/GCV/FCV at inclusion	3 (42.8)/2 (28.2)/2 (28.2)/1 (14.3)	2 (13.3)/10(66.7)/3(20)/0	.166
Baseline CMV DNAemia (IU/mL); mean ± SD	$1.44\ 10^6 \pm 2.39\ 10^5$	$1.62\ 10^6 \pm 2.56\ 10^5$	.679
Mutant strain	1 (14.3)/6(85.7)	2 (13.3)/13 (86.7)	
One year CMV DNAemia eradication	5/7 (71.4)	10/15 (66.7)	.823
One year CMV DNAemia recurrence	2/5 [40]	3/10 (30)	.387
Lymphocyte count (/μL), mean ± SD	326 ± 198	430 ± 327	.01

Abbreviations: CMV, cytomegalovirus; HR, hazard ratio; mTORi, mTOR inhibitors; SD, standard deviation.

recommended.[12] However, a cumulative (val)ganciclovir exposure exceeding 6 weeks is a strong risk factor for CMV antiviral drug resistance due to the emergence of UL97/UL54 mutations.[3,12] There is therefore an urgent need for new strategies in this scenario.

CMV recurrence is also a major issue. It is very frequent in patients with a lack of prior CMV immunity (D+/R−), a high baseline viral load (100,000 copies/mL), a failure to eradicate DNAemia by day 21 post-treatment, a weak viral load decrease during CMV therapy, or lymphopenia.[23,24] Our cohort of 63 kidney transplant recipients exhibited all of these characteristics. About 10% of our cohort exhibited CMV diseases with mutant strains which are also associated with a higher risk of recurrences.[25] Testing a new strategy to improve CMV DNAemia eradication and to prevent recurrence in these patients was therefore very relevant but also very challenging.

Secondly, the lack of mTORi conversion efficacy could be related to the absence of tacrolimus minimization. In *Transform* and *Athena* studies, the lower incidence of CMV events could be related to everolimus or to the reduced dose of tacrolimus.[9,26,27] In our study, most of the patients converted to everolimus had a tacrolimus blood concentration over 5 ng/mL because physicians are reluctant to decrease the tacrolimus trough level. Indeed, it has been associated with de novo donor-specific antibodies emergence and a lower graft survival.[28] It probably resulted in a state of over-immunosuppression which could have contributed to this absence of effect on CMV.

Thirdly, the analysis of the CMV-specific Vδ2[neg] T lymphocyte expansion following the CMV disease or recurrence showed that conversion to mTORi was not associated either with a stronger or with a faster anti-CMV immune response.[20] The protective effect of mTORi

TABLE 4b Baseline characteristics in "CMV recurrent" group

Characteristic	Conversion to mTORi, $n = 9$	No conversion to mTORi, $n = 32$	P-value
CMV status	7 (77.8)/2(22.8)	23 (71.8)/9(28.2)	.724
Preemptive versus universal prophylaxis	1 (11.1)/8(89.9)	9(28.2)/23(71.8)	.293
Early-onset versus "post-prophylaxis" CMV disease	1 (11.1)/8(89.9)	8(25)/24[75]	.999
Baseline CMV DNAemia (IU/mL); mean ± SD	$2.20\ 10^5 \pm 3.24\ 10^5$	$5.3\ 10^6 \pm 1.91\ 10^5$	.504
Mutant strain	1 (11.1)/8(89.9)	0/32	.219
One year CMV DNAemia eradication	6/9 (66.7)	30/32 (93.7)	.061
One year CMV DNAemia recurrence	2/6 (33.3)	15/30 [50]	.661
Lymphocyte count (/μL); mean ± SD	1 024 ± 848	762 ± 453	.731

This table describes the baseline CMV characteristics among patients with CMV recurrence regarding donor and recipient status, type of preventive strategy, inclusion for CMV persistence or recurrence, the antiviral treatment, virus characteristics, and lymphocyte count.
Abbreviations: CMV, cytomegalovirus; HR, hazard ratio; mTORi, mTOR inhibitors; SD, standard deviation.
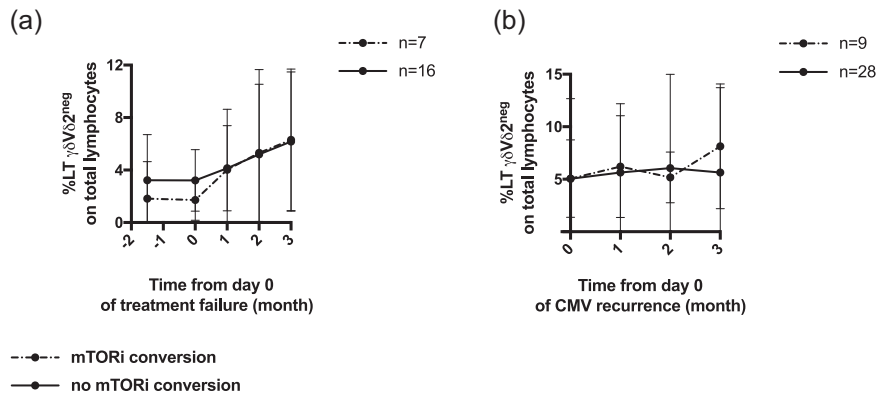
FIGURE 3 Comparison of Vδ2^{neg} T lymphocyte kinetics during persistent and recurrent CMV diseases between patients with or without mTORi conversion. (a) Comparison of Vδ2^{neg} T lymphocyte kinetics during persistent CMV diseases between patients with or without mTORi conversion. Vδ2^{neg} T lymphocyte (% of total T lymphocytes) of patients with or without mTORi conversion were compared with Mann–Whitney test on day 0 of CMV infection ($P = .329$), week 7 (called month 2) which was the date of the CMV persistence definition ($P = .186$); and every month during 3 months after week 7 (month 3, $P = .851$; month 4, $P = .622$; month 5, $P = .168$). (b) Comparison of Vδ2^{neg} T lymphocyte kinetics during recurrent CMV diseases between patients with or without mTORi conversion. Vδ2^{neg} T lymphocyte (% of total T lymphocytes) of patients with or without mTORi conversion were compared with Mann–Whitney test on day 0 of recurrent CMV disease ($P = .449$), and every month during 3 months (month 1, $P = .334$; month 2, $P = .255$; month 3, $P = .959$). CMV, cytomegalovirus; mTORi, mTOR inhibitor

against CMV could depend on the immunological status of the recipient, and mainly benefit patients with pre-formed CMV-specific immunity. Two randomized studies have shown that mTORi attenuated the incidence of CMV disease mainly in R+ patients.[10,11] At the time of transplant, most R+ patients had memory CMV-specific T cells that could control the virus.[29] mTORi may influence this immune-mediated response in R+ recipients, since it has been reported that sirolimus exerted dose-dependent immunostimulatory effects on CD8+ memory T cells in both mice and macaques exposed to viral pathogens.[7,30] More recently, an in vitro study demonstrated that mTORi significantly improved preformed CMV-specific effector memory T-cell function.[31] Here, we focused mainly on naive D+R− KTR, for whom mTORi have controversial effects.[30,32,33] From day 0 post transplantation to the end of universal prophylaxis, most of the D+R− KTR did not develop any CMV-specific T cells.[34] Moreover, persistent CMV DNAemia or recurrence is also observed in D+R− patients with a lack of CMV immunity.[22] Interestingly, mTORi did not influence naive T cells in vitro, nor their overall activation marker expression after CD3/CD28 coactivation.[31] Our clinical data also suggest an inability of mTORi to control CMV infection in naive patients.

Finally, our study has some limits. It is a retrospective and monocentric study with a low number of patients. Very few R+ recipients develop CMV persistence or recurrence. CMV diseases in these patients are usually easily manageable. It is therefore impossible to include more R+ patients displaying the chosen inclusion criteria. Even in D+R− patients, these difficult CMV scenarios (CMV persistence or CMV recurrence) are not frequent enough to include more patients.

Moreover, the absence of standardized criteria to convert patients to mTORi could introduce potential hidden bias. One may expect a trend to consider patients with more difficult CMV disease to treat as eligible for conversion to mTORi but no difference in clinical CMV parameters was observed between the two groups. Prophylactic strategies changed over time but were equally balanced between the two groups with or without mTORi conversion. The assay to monitor DNAemia also changed over time but the threshold of CMV DNAemia detectability of 250 IU/mL did not change, which allowed us to analyze equally the eradication and recurrences all over the period. We may also point out a delay in the conversion to mTORi after inclusion. However, despite these drawbacks, our study is the first clinical experiment of the use of mTORi, compared with maintaining the standard immunosuppressive regimen: mTORi were quite well tolerated with no impact on graft function, rejection, and graft survival but showed no effect in such challenging scenarios.

In conclusion, the use of mTOR inhibitors did not lead to any improvement in the outcomes of CMV disease with persistent DNAemia or recurrence in that high-risk population.

Testing the conversion of mTORi in less severe CMV diseases, in R+ patients with preformed CMV immunity, or at an earlier stage of CMV disease (as soon as the first positive CMV QNAT is detected) should be tested in the future.

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SUPPORTING INFORMATION

Additional supporting information may be found online
in the Supporting Information section.

DRAFT 1
Identification of a natural repertoire of innate-like human γδ T cells reactive to CMV

We are still working on the best way to introduce our project. Here is a first attempt of introduction together with bullets points that we would like to address. Then we present the results obtained for now and the methods used.

Introduction

Even if those criteria have been shaken, we still try to classify the immune response as "innate" and "adaptive". "Innate" defines immune actors that are rapidly present on the site of antigens, that can activate very quickly after target recognition but that are poorly specific with conserved and weakly diverse antigen receptors. Conversely, "adaptive" is a term dedicated to actors that are not firstly in the tissues, that display a diverse antigen-receptor repertoire, that needs priming and clonal expansion of antigen-specific cells prior to acquire effector functions and that develop long term antigen-specific protection called "immune memory". This memory state has been well studied for B cells, αβ T cells and more recently for NK cells in the context of CMV even if they did not express a specific antigen-receptor.

Since they express a T cell receptor to antigen, γδ T cells have consequently been frequently described by comparison of B cells and conventional αβ T cells. Like B cells, their TCR structure allowed the recognition of native antigen whereas conventional ab T cells need a processing peptide-antigen presentation within HLA molecules. Similarly to conventional αβ T cells, γδ T cells present a thymus passage where they acquire their TCR expression, γ an δ as compared to α and β for conventional T cells. All those receptors present a common ability for

"theoretical" specificity and diversity obtained by somatic gene rearrangements from a limited number of V (Variable), D (Diversity), J for junction) and C (Constant) genes and by junctional diversity with addition or deletion of nucleotides between the different gene segments when they are joined together, also called somatic hypermutations. This diversity is generated mainly in the complementary-determining region 3 (CDR3) of the T-cell antigen receptor (TCR) or B-cell antigen receptor (1) that allows a large spectrum generation of antigen-specific receptors. However, in humans, the TCR chains type and their diversity, their pre-acquired function, and their localization used to distinguish Vδ2Vγ9 T cells from the others collectively called Vδ2neg $\gamma\delta$ T cells which are those involved in CMV response.

The Vγ9^{pos}Vδ2^{pos} T cells are considered as innate-like cells and are the predominant subset in the peripheral blood. Their developmental studying is the first argue to define them as an innate subset since during fetal life, this subset had a restricted Vγ9Vδ2 TCR diversity including more than 50% of a germline-encoded sequence, with preprogrammed effector function (IFNγ, granzymes A-K) (2). Then after birth, a second thymic production wave is suggested since the TCR diversity repertoire was amplified but still remain restricted with increase N-additions (3). Their restrained TCR diversity is in linked with their restrained type of activation. Vγ9Vδ2 T cell recognized small phosphorylated intermediate metabolites of the isoprenoid synthesis pathways, also called phosphoantigens (pAg), such as isoprenyl pyrophosphate (IPP) and (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (4). Sensing of these cellular phosphoantigens by Vγ9Vδ2 T cells is mediated by interactions between members of the butyrophilin family and the Vγ9Vδ2 TCR with the intracellular B30.2 domain of BTN3A1 binding pAg (5) and the BTN2A1binding of the germline-encoded HV4 region of the Vγ9 (6, 7).

The others, Vδ2^{neg} $\gamma\delta$ T cells are the last identified actors of the cell mediated immunity against CMV i (8, 9). Even if preferentially located in epithelial tissues, they have been more described

51   as adaptive subset because of the diversity of their TCR repertoire, their delayed kinetics of

52   expansion common to the ones of CD8+ $\alpha\beta$ T cells and their acquisition of memory phenotype

53   (*10, 11*). Indeed, during CMV infection, they performed a switch from naïve to TEMRA

54   differentiation stage just like conventional $\alpha\beta$ T cells and present a clonal expansion suggesting

55   an antigen-driven recognition (*12*).

56   However, recently, human $\gamma\delta$ T cell development showed two different waves of non Vδ2 $\gamma\delta$ T

57   cells production leading both to innate-like and adaptive-like cells (*13*). Haematopoeitic stem

58   cell precursors coming from the liver were responsible of a first wave of $\gamma\delta$ T cell production

59   and lead to the generation of effector invariant germline-encoded $\gamma\delta$ T cells that were

60   functionally programed (IFNγ and Granzyme B) (*13*) and showed CMV-reactivity. After birth

61   and in adult life, $\gamma\delta$ production come from hematopoeitic stem cell precursors of the bone

62   marrow and lead to polyclonal cells without pre-established functions (*13*) associated with

63   naïve phenotype (*14*). Consequently, both invariant innate-like and polyclonal non Vγ9Vδ2 T

64   cells can be produced and are CMV-reactive.

65   The second argue for innate-like properties relies on their tissue localization and kinetic of

66   response during CMV that are difficult to appraise in human and but for which data are available

67   in mice (*15*). Indeed, during the course of CMV infection in human as previously said, kinetic

68   of expansion has been parallel to those of $\alpha\beta$ T cells but their preferential tissue localization

69   would suggest an earlier intervention. Even if not required to control infection in a wild-type

70   mouse, $\gamma\delta$ T cells permit to protect CD3ϵ KO and Rag$^{-/-}$ γc$^{-/-}$ mice from CMV-induced death

71   (*15*) and were into the tissues and produced IFNγ from day 3 of CMV infection (perhaps earlier

72   but have not been checked before) (*15*). Those observations participate to attribute innate-like

73   characteristics to $\gamma\delta$ T cells during CMV infection unlike their TCR diversity (*16*).

74   Consequently, the presupposed innate-like properties of $\gamma\delta$ T cells for responding fastly to CMV

75   combined with their already well-described adaptive characteristics with long-term memory

76   instauration lead to focus on them among the arsenal of CMV immune response. We proposed

77   in this work to evaluate the innate-like CMV-abilities and repertoire of naïve Vδ2^{neg} $\gamma\delta$ T cells

78   in CMV negative individuals, that could constitute a natural $\gamma\delta$ T cell repertoire to CMV.

79

80

Bullet points under consideration:

Rational to explore innate features of CMV-responding γδ T cells:

- 1. Shaken and evolutive definition of "innate" and "adaptive" in immunology. To which cells it refers? (myeloid, innate lymphoid cells, NK cells= innate cells *versus* T and B cells = adaptive). This innate and adaptive notion have been recently extended to T cells (and B cells also): to conventional adaptive αβ T cells are opposed the "innate-like" or "unconventional T cells" (prototype: NKT cells, but also MAIT cells, and γδ T cells). They are also called "transitional" T cells, meaning: in between innate and adaptive cells.

- 2. Our definition of "innate-like T cells" and "adaptive-like T cells".

  - Innate-like" T:
    - Antigen (Ag) recognition: Do not recognize processed pathogen-derived peptides presented by polymorphic classical MHC molecules.
    - Less specific process of Ag recognition, closer to PAMPs or DAMPs recognition by PPR on myeloid cells; based on invariant or semi-invariant TCRs expressed by large subsets and recognizing non-polymorphic molecules (MHC-like or not).
    - Independent TCR activation (eg NKG2D).
    - Localization: generally within tissues but some can circulate such as Vγ9Vδ2.

  - "Adaptive" T:
    - Ag recognition: recognize processed pathogen-derived peptides presented by polymorphic classical MHC molecules; highly specific of a given pathogen.
    - Activation: priming of naïve cells that acquire effector functions after clonal expansion; focusing of the TCR repertoire toward the pathogen.
    - Memory: long-term and specific protection against the pathogen = immune memory.
    -

113   o   Adaptive-like" T cells: should we introduce this notion that is applied to T cells
114   considered as "innate-like" but which finally seem to be more adaptive than
115   expected (typically the CMV-reactive γδ T cells). Shift in the paradigm?

116

117   •   3. Focus on γδ T cells:
118   o   they have traditionally been place in the "innate-like" T cells because the subsets
119   that have been the most studied in mice (TCR invariant DETC cells) and in
120   human (semi-invariant Vγ9Vδ2 T cells) have obvious "innate-like" properties.
121   o   However, due to the large diversity and heterogeneity of the different subsets
122   composing γδ T cells, this classification is being revised.

123

124   •   4. Elements for the adaptive features of γδ T cells:

125

126   o   While they have long been considered as unable to develop memory response,
127   several papers have recently demonstrated that some γδ subsets can confer long-
128   term protection (Leo Lefrançois) and during CMV infection.
129   Immunophenotyping of patients also give argues for memory function of γδ T
130   cells notably a faster expansion during reinfection.
131   o   Clonal expansion in the context of CMV and kinetics like αβ T cells
132   o   Effector/memory phenotype in diverse situations

133

134   •   5. Elements for the innate feature of γδ T cells response:
135   o   In mice model : their fast localization in tissues and their fast IFNγ production
136   o   In patients : their massive expansion, rare other pathogen situation in which they
137   expand

138

Results

Phenotypic characterization of naive Vδ2^{neg} $\gamma\delta$ T cells

Naive $\gamma\delta$ T cells have been poorly characterized in human because the main subset, namely the Vγ9Vδ2 T cells globally acquire a effector/memory phenotype in early life, and Vδ2^{neg} $\gamma\delta$ T cells are highly differentiated in CMV-seropositive donors. The latter are naive in CMV-negative donors but represented at very low numbers. The proportion of CD27+CD45RA+ Vδ2^{neg} $\gamma\delta$ T cell are indeed the largest majority in CMV naive individualswhereas CD27-CD45RA+ Vδ2^{neg} $\gamma\delta$ T cell are the largest majority in CMV positive donors (**Figure 1A**). However, other markers could contribute to define more precisely naive cells We compared those markers among CD45RA+CD27+ $\alpha\beta$ and Vδ2^{neg} $\gamma\delta$ T cells among the same CMV-negative individual. As shown in Figure 1B, both subsets of CD45RA+CD27+ cells among $\alpha\beta$ and $\gamma\delta$, are mainly CCR7+, CD28+, CD11a intermediate (CD11a dull and CD45RO- (**Figure 1B**).

Further characterization of naïve versus effector/memory $\gamma\delta$ cells by scRNASeq (to be analyzed).

CD45RA+CD27+ Vδ2^{neg} $\gamma\delta$ T cells present a fast and specific reactivity against CMV infected cells

We next tested the CMV-reactivity of this naïve population of Vδ2^{neg} $\gamma\delta$ T cells from CMV-negative donors, in comparison with the reactivity of TEMRA Vδ2^{neg} $\gamma\delta$ T cell from a CMV-positive donors. For this purpose, we either sorted naïve Vδ2^{neg} $\gamma\delta$ T cell from CMV-negative donors or TEMRA Vδ2^{neg} $\gamma\delta$ T cell from CMV-positive donors (gating on **Figure 2A**) and measured their production of IFNγ in vitro when cultured in media alone, or with non-infected or CMV-infected fibroblast during a short period of 24 hours. IL18 was added to the cultures

as we have previously shown that it amplifies the $\gamma\delta$ TCR induced production of IFNγ (Guerville et al). We observed that naïve V$\delta2^{neg}$ $\gamma\delta$ T cells from CMV-negative donors (either adult or cord-blood) produced a significant amount of IFNγ when co-cultured with CMV-infected fibroblasts as compared to medium alone or to non-infected fibroblast., Even though, this production of IFNγ was expectedly lower than that of TEMRA V$\delta2^{neg}$ $\gamma\delta$ T cells from CMV-positive donors, the ratio of IFNγ production between CMV-infected and non-infected fibroblasts is equivalent for TEMRA and naïve V$\delta2^{neg}$ $\gamma\delta$ T cells (**Figure 2B and 2C**). Interestingly, by contrast to naïve V$\delta2$neg $\gamma\delta$ T cells, TEMRA V$\delta2$neg $\gamma\delta$ T cells from CMV-negative donors did not produce IFNγ in the presence of CMV-infected cells. This result suggests that TEMRA cells in CMV-negative donors have been selected by other pathogens or antigens and are not able to respond to CMV, also strengthening the specificity of our assay. . Conversely in CMV-positive donors with enough naïve V$\delta2^{neg}$ $\gamma\delta$ T cells to allow sorting of enough cells, both naïve and TEMRA V$\delta2^{neg}$ $\gamma\delta$ T cell reacted to CMV-infected fibroblast (**Figure 2C**).

Moreover, the specificity of V$\delta2^{neg}$ $\gamma\delta$ T cells to CMV was evaluated by testing IFNγ production by naïve V$\delta2^{neg}$ $\gamma\delta$ T cell in culture with fibroblast infected either with two other herpesviruses, HSV or VZV. We observed that naïve V$\delta2^{neg}$ $\gamma\delta$ T cells from cord-blood or from adults negative for CMV presented IFNγ production only in co-culture with CMV-infected fibroblasts and not with HSV or VZV-infected fibroblasts (**Figure 2D**). This activation was not driven by non-specific signals (for instance through the release of cytokines) that could activate any type of lymphocytes since naïve ab T cell from the same CMV negative donors did not produce IFNγ when cultured with CMV-infected fibroblasts (**Figure 2E**).

Altogether these results demonstrated a rapid and specific activation of naïve V$\delta2$neg $\gamma\delta$ T cells against CMV-infected cells.

188

Innate-like activation of naïve Vδ2neg γδ T cells **against CMV.**

The very quick capacity of naïve Vδ2neg γδ T cells to produce IFNγ in 24h without the

requirement of prior priming was kind of a surprise, and suggested an innate-like type of

activation. We thus compared the activation kinetics of naive γδ and αβ T cells against CMV.

We used a mix of peptides from the CMV pp65 tegument protein to activate CMV-specific ab

T cells within PBMC, and used the activation markers CD69 and CD71 as a read-out of

response. In parallel, negatively sorted γδ T cells from the same donor were cultured with

CMV-infected fibroblasts. We performed those experiments with and without IL18. . We

observed that naïve ab T cells did not express CD71-CD69 after 24 hours of activation with

pp65 peptides, with or without IL18 activation (**Figure 3A**). However, naive γδ T cells present

an increase CD69/CD71 double positive population compared to condition in media alone and

with non-infected fibroblast with or without IL18 (**Figure 3B**).

These results showed that naïve Vδ2^{neg} γδ T cells of CMV-negative donors have a specific

ability to promptly respond to CMV-infected cells with a much more rapid kinetics than ab T

cells, reminiscent of an innate-like type of response..

204

205

Analysis of the CMV-responding naïve Vδ2^{neg} γδ T cell proportion.

Then, we wondered what was the proportion of naïve Vδ2^{neg} γδ T cells able to respond to CMV-infected cells .

In a first place, we performed an IFNγ ELISPOT assay on Vδ2^{neg} γδ T cells that were activated with uninfected or CMV-infected cells, or with PMA and ionomycin as positive control. In two CMV-negative donors, we observed that 0.26 and 0.23% of naïve Vδ2^{neg} γδ T cells produced IFNγ specifically on CMV-infected cells (**Figure 4A**). To extend the analysis of CMV-responding naïve Vδ2^{neg} γδ T cell repertoire beyond those producing IFNγ, we next analyzed the proportion of positive cells for two activation markers namely CD69 and CD71. Without IL18, this percentage of double positive CD69/CD71 cells was in average 0.58% and 2.2% with IL18 (**Figure 3B**) on four different donors.

To be even more general and avoid usage of activation marker on CMV-responding naïve Vδ2^{neg} γδ T cell, we finally used the unbiased method of single-cell RNA sequencing. After an overnight activation with non-infected or CMV-infected fibroblasts in the presence of IL18, IFNγ ELISA was performed to confirm activation (**Supplemental Figure 1**) and naïve Vδ2^{neg} γδ T cell were analyzed by UMAP after RNA single-cell sequencing (**Figure 4B**). When mixing the data obtained from γδ T cells harvested from the co-culture with uninfected and with CMV-infected fibroblasts, this unbiased analysis allowed the identification of a cluster only constituted of naïve Vδ2^{neg} γδ T cell coming from the culture with CMV-infected fibroblasts that represent 35% of all naïve Vδ2^{neg} γδ T cells of this condition, suggesting a very large amount of CMV-responding naïve Vδ2^{neg} γδ T cells. Focusing on IFNγ expressing cells, we also found a 0.2% of positive cells in the CMV-infected cells, confirming the data obtained using the IFNγ ELISPOT.

Analysis of TCR repertoire

We would like to perform the analysis of the TCR repertoire of those CMV-responding naïve Vδ2^{neg} γδ T cell. For this purpose, we will compare by NGS sequencing the diversity of Vd and Vg chains between double negative and double positive CD69/CD71 cells of Vδ2^{neg} γδ T cells after 24 hours of culture with non-infected and CMV-infected fibroblasts to see if positive cells during CMV activation are more or similarly clonal than the double negative ones (in progress in David Vermijlen's team).

Signature and functions of CMV-responding naïve Vδ2^{neg} γδ T cell

To go further in the characterization of CMV-responding naïve Vδ2^{neg} γδ T cell signature, we analyzed the expression of highly expressed and highly downregulated molecules in the cluster only constituted of naïve Vδ2^{neg} γδ T cell coming from culture with CMV-infected fibroblasts (cluster 1) compared to all-other (cluster 2) (**Figure 5**). We observed that IL18RAP (coding for Interleukin-18 receptor accessory protein involved in IL18-dependent transduction signal), CFS2 (coding for GM-CSF), CD160, CCR1 (receptor of CCL3 /MIP-1 α, CCL5/RANTES), CCL7/MCP-3 and CCL23/MPIF-1), IFNγ and TNFSF9 (CD137) were ones of the more overexpressed genes in cluster 1. Conversely, CCR7 was one of the most downregulated, suggesting that CMV-responding naïve Vδ2^{neg} γδ T cell have evolved into a maturation process compared to the others. We will further analyze this signature and confirm at a protein level the most relevant receptors and molecules belonging to this signature.

We will extend thus the functions of CMV-responding naïve Vδ2^{neg} γδ T cells since for now, IFNγ was the most studied cytokine involved in the reactivity of Vδ2^{neg} γδ T cells.

Recognition mechanisms involved in naïve Vδ2^{neg} γδ T cell reactivity against CMV-infected cells

255 The clonal expansion of Vδ2^{neg} γδ T cells observed during CMV infection (*19*) suggests an

256 antigen-driven recognition and raises the question of TCR involvement in the naïve Vδ2^{neg} γδ

257 T cells against CMV-infection. To test TCR involvement in the naïve γδ T cells recognition of

258 CMV-infected cells, we first performed the same coculture with sorted γδ T cells from CMV

259 negative donors and media alone, non-infected fibroblasts and CMV-infected fibroblasts with

260 and without anti-CD3 (UCHT1) and observed a weak decrease of double positive CD69/CD71

261 in only two donors. So, these results have to be confirmed and controlled with other antibodies

262 (ie Isotype). The TCR involvement will also be tested by deleting the Cd gene by CRISPR-

263 Cas9 to definitively attest the necessity of the TCR engagement in the CMV-reactivity observed

264 of naïve Vδ2^{neg} γδ T cells. Since we observed a higher reactivity with IL18 compared without

265 IL18 , and also observed that IL18RAP was upregulated in the cluster 1 of naïve Vδ2^{neg} γδ T

266 cells, we will phenotype naïve Vδ2^{neg} γδ T cells for the expression of IL18 receptor before and

267 after culture with CMV-infected cells.

268 Moreover, NKG2D is also one of the major receptor on γδ T cells involved in their activation

269 (*20*), consequently we will also test its involvement in naïve Vδ2^{neg} γδ T cells reactivity against

270 CMV by antibodies blocking.

271

Materials and Methods

274 For complete reference of antibodies, see supplemental Methods.

Sample and Patients

276 We used PBMC from healthy blood-donor of EFS (Établissement Français du Sang) for whom

277 CMV serology was assessed. Before experiment where PBMC have to be sorted for activation,

278 flow cytometry phenotyping of donors was performed on whole blood as previously described

279   (53) using the following monoclonal antibodies: anti-CD3 V450, anti-panδ-PE, anti-Vδ2-PC7,

280   anti-CD27 APC and anti-CD45RA-FITC and with the Lysing Solution IOTest®3 10X

281   Concentrate (Beckman) to have pre-sorted staining. The samples were processed on a canto II

282   cytometer (BD Biosciences).

283

284   **Phenotyping of γδ T cells and αβ T cells of CMV negative donors.**

285   One million frozen PBMC were used and stained with the viability marker FVS575,

286   monoclonal antibodies against CD3 (BV510), Vδ2 (PC-7), pan-delta (PE), CD45RA (BV786),

287   CD27 (BV650); CD11a (FITC), CD28 (APC), CCR7 (BV421), and CD45R0 (APC-H7). Then,

288   PBMC were washed and resuspended in PBS supplemented with 0.1% bovine serum albumin

289   and 2mM EDTA. Stained cells were processed on the BD LSR Fortessa (BD biosciences).

290

291   **CD69/CD71 staining of γδ T cells and αβ T cells from CMV negative donors**

292   From the same donors, whole PBMC were kept for αβ T cell activation and negative magnetic

293   sorting with pan T cell isolation kit; αβ–biotin and anti-biotin (Miltenyi Biotec), Vδ2 FITC

294   (Beckman) anti-FITC (Miltenyi Biotec) was performed for γδ T cell activation. One million of

295   PBMC for αβ T cell activation were incubated in 96 well plates with 150μL of the previously

296   described media with or without IL18 in medium alone, 0.6 nmol/mL of peptivator CMV pp65

297   (130-093-438, Miltenyi) and γδ T cells with non-infected or CMV-infected fibroblasts for 24

298   hours , or PMA 25ng/ml/ionomycine 1μg/ml (Sigma) for 5 hours at 37°C and then cells were

299   stained with anti-CD3 (V450), CD27 (BV650), CD45RA (FITC), CD27 (APC), pan-γδ (PE),

300   CD69 (AF700) and CD71 (BV786) monoclonal antibodies. For TCR blocking experiments,

301   anti-CD3-V450 was incubated with γδ T cells for 15 minutes in PBS at 4°C, washed, and γδ T

302   cells were incubated as previously described overnight of culture, and stained with the same

303   panel. All PBMC from each sample were processed by BD LSRFortessa (BD biosciences).

304

305   **Sorting naive and TEMRA γδ T cells or naïve αβ T cells before culture with fibroblasts**

306   **and IFNγ measurement.**

307   Negative magnetic sorting with pan T cell isolation kit; αβ–biotin and anti-biotin (Miltenyi

308   Biotec), Vδ2 FITC (Beckman) and anti-FITC (Miltenyi Biotec). Then, PBMC were stained

309   with anti-CD3 V450, anti-panαβ-PE, anti-Vδ2-PC7, anti-CD27 APC and anti-CD45RA-FITC

310   and either CD3+ αβ–Vδ2- CD27+ CD45RA+ or CD45RA- or both were purified. 10 000 cells

311   were incubated *per* 96 wells-plate with RPMI 8% FCS, 2mM glutamine and 50ng/ml rIL18

312   (MBL international, B003-5, Massachusetts-US) (see (57)) alone, or with either non-infected

313   or CMV/HSV/VZV-infected fibroblasts during 24 hours at 37°C before collecting the

314   supernatants for IFN-γ ELISA operated as described in the kit (Human IFN-γ ELISA

315   development kit, Mabtech, n°3420-1H-6). Alternatively, the γδ T cells were transferred

316   additionally overnight on the ELISPOT plate and the last steps of IFN-γ–ELISPOT were

317   performed as described in the kit (3420-4APT-2, Human IFN-γ ELISpot [PLUS] (ALP) Mabtech).

318   Naive αβ T cells were negatively sorted with pan T cell isolation kit; γδ T cell kit (Miltenyi

319   Biotec), Vδ2 FITC (Beckman) and anti-FITC (Miltenyi Biotec). Then, PBMC were stained

320   with anti-CD3 V450, anti-panγδ-PE, anti-Vδ2-PC7, anti-CD27 APC and anti-CD45RA-FITC

321   and CD3+ αβ–Vδ2- CD27+ CD45RA+ were purified. Then 10 000 cells were used per 96

322   wells-plate and culture and IFNγ measurement were performed as for γδ T cells. CMV

323

324   **Single cell RNA sequencing**

325   Single-cell libraries were generated using the Chromium Controller Single-Cell Instrument and

326   Chromium Single Cell 3′ Library & Gel Bead Kit v2 A Chip Kit and i7 Multiplex Kit (10x

327   Genomics) according to the manufacturer's protocol. Briefly, γδ T lymphocytes from a CMV

328   negative healthy donor were sorted with magnetic method as earlier described. Then 50 000

329   cells per 96 wells-plate were incubated with rIL18 50ng/ml in media alone(duplicate) and with

330   non-infected and CMV-infected fibroblast (six wells for each conditions) overnight at 37°C.

331   Then ELISA IFNγ was performed to assessed reactivity (**Supplemental Figure 1**). γδ T

332   lymphocytes were then sorted by with the BD FACS Aria IIu Cell sorter, and a 98-100 % purity

333   was obtained. Then cells were diluted to ~1000 cells/μl in 0.04% BSA–PBS. About 20,000

334   cells were added to each channel (B1: 20 000 γδ T cell from culture with non-infected

335   fibroblasts, first replicate; B2: 20 000 γδ T cell from culture with non-infected fibroblasts,

336   second replicate; B3: 20 000 γδ T cell from culture with CMV-infected fibroblasts, first

337   replicate; B4: 20 000 γδ T cell from culture with CMV-infected fibroblasts, second replicate)

338   and samples were run according to manufacturer's protocol with a targeted cell recovery

339   estimate of 8000 cells in total. After generation of nanoliter-scale Gel bead-inEMulsions

340   (GEMs), GEMs were reverse transcribed in a C1000 Touch Thermal Cycler (Bio-Rad)

341   programed at 53°C for 45 min, 85°C for 5 min, and hold at 4°C. After reverse transcription,

342   single-cell droplets were broken and the single-strand cDNA was isolated and cleaned with

343   Cleanup Mix containing Dynabeads MyOne SILANE (Thermo Fisher Scientific). cDNA was

344   then amplified with a C1000 Touch Thermal Cycler programed at 98°C for 3 min, 14 cycles of

345   (98°C for 15 s, 67°C for 20 s, 72°C for 1 min), 72°C for 1 min, and hold at 4°C. Subsequently,

346   the amplified cDNA was fragmented, end-repaired, A-tailed and adaptor ligated, with

347   SPRIselect Reagent Kit (Beckman Coulter) with cleanup in between steps. Post-ligation

348   product was amplified and indexed with a C1000 Touch Thermal Cycler programed at 98°C

349   for 45 s, 14 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 20 s), 72°C for 1 min, and hold at

350 4°C. The sequencing-ready library was cleaned up with SPRIselect beads prior to sequencing

351 on a HiSeq 3000 instrument (Illumina). The output bcl2 file was converted to FASTQ format

352 by using cellranger-mkfastq™ algorithm (10x Genomics), and cellranger-count was used to

353 align to the GRCh38 transcriptome and build the (cell, UMI) expression matrix for each sample.

354

355 **Single cell RNA-seq analyses**
356

357 This yielded for B1: n: 8,700 Estimated Number of Cells; 20,758 Mean Reads per Cell; 1,861

358 Median Genes per Cell; B2: 8,744 Estimated Number of Cells; 22,032 Mean Reads per Cell;

359 1,876 Median Genes per Cell; B3: 8,034 Estimated Number of Cells; 25,298 Mean Reads per

360 Cell; 1,965 Median Genes per Cell and B4 : 7,923 Estimated Number of Cells; 22,823 Mean

361 Reads per Cell; 1,927 Median Genes per Cell.

362 Then an UMAP analysis was performed on the entire cells and the entire genes sequenced

363 (**Figure 4 B)**, allowing to observe a cluster (cluster 1) in which only $\gamma\delta$ T cell from culture with

364 CMV-infected fibroblasts (B3 and B4) were found together conversely to all others cells on the

365 UMAP (put together as cluster 2). Secondly, single-cell-virtual-master online software allowed

366 us to gate separately cluster 1 and cluster 2 and a volcano-plot was performed to observe

367 differential gene expression of cluster one compared to cluster 2 (**Figure 5**).

368

369 **Analysis of TCR repertoire**

370 Double negative and double positive CD69/CD71 cells of naive $V\delta2^{neg}$ $\gamma\delta$ T cell after 24 hours

371 of culture with non-infected and CMV-infected fibroblasts were sorted with the BD FACS Aria

372 IIu Cell sorter after staining with -CD3 (V450), anti-pan$\gamma\delta$ (PE), anti-Vδ2 (PC7), anti-CD27

373 (APC) and anti-CD45RA (FITC-, anti-CD69 (AF700) and anti-CD71 (BV786). Then, cells

374 were counted (Neubauer) and were diluted to ~1 cell/ 2µl of RLT (n° 79216, Quiagen). Then,

375 samples were sent for NGS sequencing of Vδ and Vγ chains.

376

Preparation of CMV-, HSV- or VZV- infected fibroblasts

378 Human foreskin fibroblasts (HFF, provided by Dr H. Rezvani, INSERM, U1035), grown in

379 DMEM 8% FCS 2mM glutamine, were infected with the TB42/E, the KOS [*Herpes simplex*

380 *virus*-1 (HSV-1)] or the OKA [(*Varicella Zoster Virus* (VZV)] at a multiplicity of infection

381 (MOI) of 0.1 per cell. After virus adsorption overnight at 37°C, cells were washed and covered

382 with fresh growth medium. When cytopathic effects were ≥90% (7 days after infection for VZV

383 (with observable viral foci), 4 days after infection for CMV and 24 hours after infection for

384 HSV), cells were washed with fresh medium. Non infected (NI) cells grown in parallel were

385 mock-infected using medium alone.

386

Preparation of free CMV/HSV/VZV.

388 To produce free CMV (TB42/E strain), human foreskin fibroblasts were infected at a MOI of

389 0.1 and incubated at 37°C in culture medium DMEM, 8% bovine serum and glutamine for 10

390 days or until cytopathic effects were ≥90%. The supernatant was stored at -80°C. The

391 preparation had a titer of 2,5 10^6 PFU (plaque-forming unit)/ml, the titration was performed as

392 previously described [4].

393 To produce free HSV (KOS strain, HSV1) and VZV (OKA strain; both viruses were kindly

394 provided by Dr Sonia Burrel, Virology Unit, Pitié Salpétrière University Hospital), MRC-5

395 cells (Eurobio, France) in T25cm^2 were infected with 500µL of strain and incubated at 37°C.

396 After 24 hours, the supernatant was removed and replaced with fresh DMEM containing 8%

397 FCS and 2mM glutamine. This operation was iterated 72 hours later. A control was prepared

398 as follows: MRC-5 cells were mock-infected and incubated following the same protocol.

399   For HSV, the supernatant was collected when cytopathic effect was of 50-100%, and mixed

400   with 50% final of FCS and then stored at -80°C. The preparation had a titer of 8.10^6 PFU/ml.

401   For VZV, the supernatant and the infected cells were collected without trypsin and used to

402   infect new MRC5 cells until at least 5 viral foci could be observed. The supernatant and the

403   infected cells were finally collected and mixed in a final 10% DMS0, 20% FBS solution and

404   stored at -80°C. The preparation had a titer of 9.10^3 PFU/ml. HSV and VZV titrations were

405   performed as previously described [5]. All virus stocks and cells were negative for the presence

406   of mycoplasma.

407

Statistics

409   The Mann-Whitney U test, the χ^2 test or the Fisher-test, the unpaired t test were used when

410   appropriate. $p < 0.05$ was considered statistically significant. Alternatively, paired-t test was

411   used for paired data. Analyses were performed with conventional statistical methods using

412   GraphPad Prism. Figures were obtained with FlowJo software (V.10) and GraphPad Prism

413   software (version 6.0, GraphPad Software, San Diego, California). RStudio statistical software

414   (version 1.1.423) was used to perform UMAP analysis.

Figure legends

Figure 1 CD45RA+CD27+ Vδ2^{neg} γδ T cell present an extended naïve phenotype in CMV negative donor

Vδ2^{neg} γδ T cells and their expression of CD27 and CD45RA were analyzed by flow cytometry in healthy donors seropositive for CMV or seronegative for CMV- (CMV serology was performed at the time of immunophenotyping) for one representative donor of each (A). Extended phenotype (CD28, CCR7, CD45RO, CD11a) was additionally performed by staining one million of PBMC of CMV negative healthy donors to better characterize CD45RA+CD27+ Vδ2^{neg} γδ T cells and αβ T cells (red) among total lymphocytes (black) (B) (n=5).

Figure 2 CD45RA+CD27+ Vδ2^{neg} γδ T cell present a fast and specific reactivity against CMV-infected cells

Naive (CD27+CD45RA+) Vδ2^{neg} γδ T cells from CMV negative donors (n=4) and terminally effector memory RA+ (TEMRA, CD27-CD45RA+) Vδ2^{neg} γδ T cells from CMV positive donors (n=4) (A) or Naive (CD27+CD45RA+) and terminally effector memory RA+ (TEMRA, CD27-CD45RA+) Vδ2^{neg} γδ T cells from CMV negative donors (n=4) and CMV positive donors (n=3) (B) were purified and incubated with rIL18 in medium alone or with non-infected or CMV-infected fibroblasts for 24 hours at 37°C and IFNγ was measured by ELISA.

C. Naive (CD27+CD45RA+) Vδ2^{neg} γδ T cells from CMV negative adults (n=3) and cord blood (n=3) were purified and incubated with rIL18 in medium alone or with non-infected or CMV.HSV/VZV-infected fibroblasts for 24 hours at 37°C and IFNγ was measured by ELISA.

D. Naive (CD27+CD45RA+) Vδ2^{neg} γδ and αβ T cells from CMV negative adults (n=2) were purified and incubated with rIL18 in medium alone or with non-infected or CMV-infected fibroblasts for 24 hours at 37°C and IFNγ was measured by ELISA.

Figure 3 Direct comparison of reactivity against CMV by CD45RA+CD27+ Vδ2^{neg} $\gamma\delta$ T cells and $\alpha\beta$ T cells from CMV negative donors

A. PBMC from CMV negative donors were incubated with (n=2) or without (n=1) rIL18 in medium alone, with pp65-peptivator for 24 hours or with PMA 25ng/ml-1µg/ml ionomycin PMA/iono) for 5 hours at 37°C and were stained in order to gate CD45RA+CD27+ $\alpha\beta$ T cells and analyze their CD69 and CD71 expression by flow cytometry.

B. PBMC from CMV negative donors (n=4) were negatively sorted with magnetic bead to purify Vδ2^{neg} $\gamma\delta$ T cells. Then, cells were incubated with or without rIL18 in medium alone or with non-infected or CMV-infected fibroblasts for 24 hours at 37°C and were stained in order to gate CD45RA+CD27+ Vδ2^{neg} $\gamma\delta$ T cells and analyze their CD69 and CD71 expression by flow cytometry.

Figure 4 Analysis of the CMV-responding naive Vδ2^{neg} $\gamma\delta$ T cell proportion

A. Proportion of IFNγ-producing Vδ2^{neg} $\gamma\delta$ T cells was assessed by IFNγ ELISPOT in two CMV negative donors (Figure representative for one donor). Vδ2^{neg} $\gamma\delta$ T cells were purified with ARIA sorter and incubated with rIL18 either in media alone (simplicate), PMA 25ng/ml-1µg/ml ionomycin (simplicate) or non-infected or CMV-infected fibroblast (duplicates).

B. Cluster of CMV-responding naive Vδ2^{neg} $\gamma\delta$ T cells was assessed by UMAP analysis after RNA single cell sequencing of purified naive Vδ2^{neg} $\gamma\delta$ T cells either incubated with non-infected (green) or CMV-infected (red) fibroblasts. One experiment has been performed.

Figure 5 Analysis of the CMV-responding naive Vδ2^{neg} $\gamma\delta$ T cell signature

UMAP analysis was performed on the entire cells and the entire genes sequenced (**Figure 4 B**), allowing to observe a cluster (cluster 1) in which only $\gamma\delta$ T cell from culture with CMV-infected fibroblasts were found together conversely to all others cells on the UMAP (put together as

464   cluster 2). Gating separately cluster 1 and cluster 2 with Single-cell-virtual-master online

465   software was performed and a volcano-plot allowed to observe the differential gene expression

466   of cluster one compared to cluster 2. Those results are obtained form single cell analysis of one

467   experiment.

468

469   **Figure 6 TCR involvement of naïve Vδ2^{neg} $\gamma\delta$ T cell for their reactivity against CMV-**

470   **infected cells.**

471   PBMC from CMV negative donors (n=2) were negatively sorted with magnetic bead to purify

472   Vδ2^{neg} $\gamma\delta$ T cells. Then, cells were incubated with rIL18 in medium alone or with non-infected

473   or CMV-infected fibroblast for 24 hours at 37°C. Moreover, for TCR-blocking, anti-CD3-V450

474   was incubated with cells for 15 minutes in PBS at 4°C, washed and incubated with rIL18 and

475   CMV-infected cells. Then, cells were stained in order to gate CD45RA+CD27+ Vδ2^{neg} $\gamma\delta$ T

476   cells and analyze their CD69 and CD71 expression by flow cytometry. Example of CD69/CD71

477   gating of CD45RA+CD27+ Vδ2^{neg} $\gamma\delta$ T cells in one donor (A) and histograms for two donors

478   (B).

479

References

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Figure 1

A.
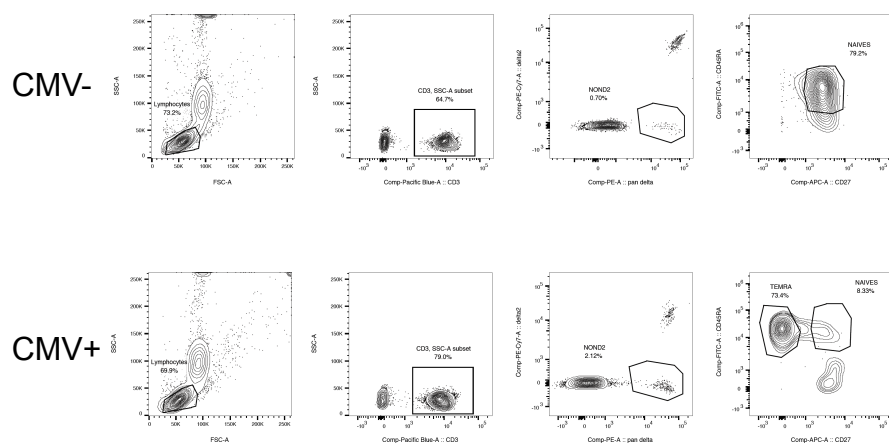
B. Among CD45RA+CD27+ T cells in CMV- donor

among Vδ2^neg γδ T cells   among αβ T cells
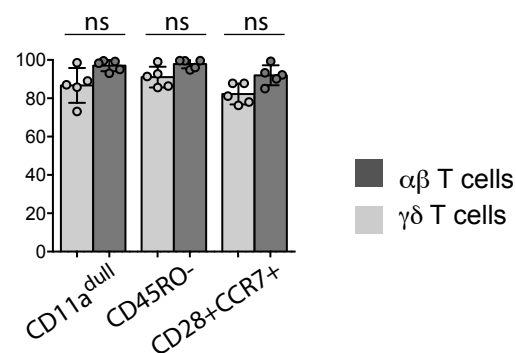
■ total lymphocytes
■ specific T cells

B. Among CD45RA+CD27+ T cells in CMV- donor

■ αβ T cells
■ γδ T cells

Figure 1 CD45RA+CD27+ Vδ2^neg γδ T cell present an extended naïve phenotype in CMV negative donor

Vδ2^neg γδ T cells and their expression of CD27 and CD45RA were analyzed by flow cytometry in healthy donors seropositive for CMV or seronegative for CMV- (CMV serology was performed at the time of immunophenotyping) for one representative donor of each (A). Extended phenotype (CD28, CCR7, CD45RO, CD11a) was additionally performed by staining one million of PBMC of CMV negative healthy donors to better characterize CD45RA+CD27+ Vδ2^neg γδ T cells and αβ T cells (red) among total lymphocytes (black)(B).

Figure 2

Figure 2 CD45RA+CD27+ Vδ2^{neg} γδ T cell present a fast and specific reactivity against CMV-infected cells

Naive (CD27+CD45RA+) Vδ2^{neg} γδ T cells from CMV negative donors (n=4) and terminally effector memory RA+ (TEMRA, CD27-CD45RA+) Vδ2^{neg} γδ T cells from CMV positive donors (n=4) (A) or Naive (CD27+CD45RA+) and terminally effector memory RA+ (TEMRA, CD27-CD45RA+) Vδ2^{neg} γδ T cells from CMV negative donors (n=4) and CMV positive donors (n=3) (B) were purified and incubated with rIL18 in medium alone or with non-infected or CMV-infected fibroblasts for 24 hours at 37°C and IFNγ was measured by ELISA.

C. Naive (CD27+CD45RA+) Vδ2^{neg} γδ T cells from CMV negative adults (n=3) and cord blood (n=3) were purified and incubated with rIL18 in medium alone or with non-infected or CMV/HSV/VZV-infected fibroblasts for 24 hours at 37°C and IFNγ was measured by ELISA.

D. Naive (CD27+CD45RA+) Vδ2^{neg} γδ and αβ T cells from CMV negative adults (n=2) were purified and incubated with rIL18 in medium alone or with non-infected or CMV-infected fibroblasts for 24 hours at 37°C and IFNγ was measured by ELISA. NI, non-infected

Figure 3
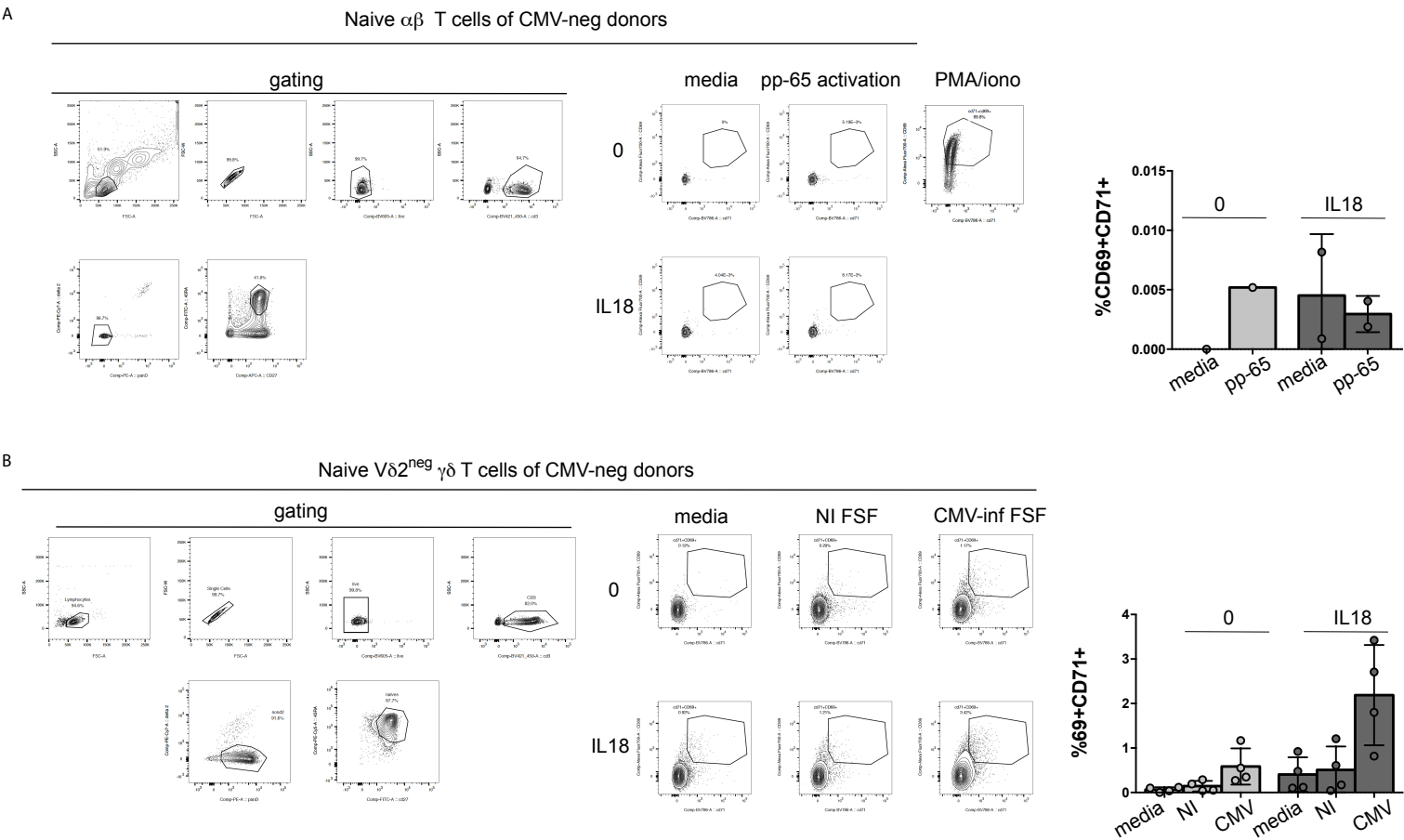
Figure 3 Direct comparison of reactivity against CMV by CD45RA+CD27+ Vδ2^{neg}γδ T cells and αβ T cells from CMV negative donors

A. PBMC from CMV negative donors were incubated with (n=2) or without (n=1) rIL18 in medium alone or with pp65-peptivator for 24 hours at 37°C and were stained in order to gate CD45RA+CD27+ αβ T cells and analyze their CD69 and CD71 expression by flow cytometry.

B. PBMC from CMV negative donors (n=4) were negatively sorted with magnetic bead to purify Vδ2^{neg}γδ T cells. Then, cells were incubated with or without rIL18 in medium alone or with non-infected or CMV-infected fibroblats for 24 hours at 37°C and were stained in order to gate CD45RA+CD27+ Vδ2^{neg} γδ T cells and analyze their CD69 and CD71 expression by flow cytometry. NI, non-infected fibroblasts

Figure 4
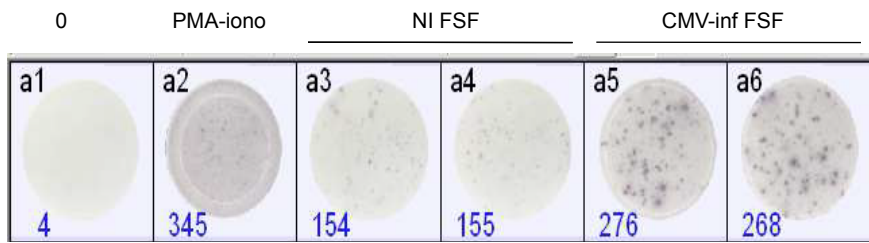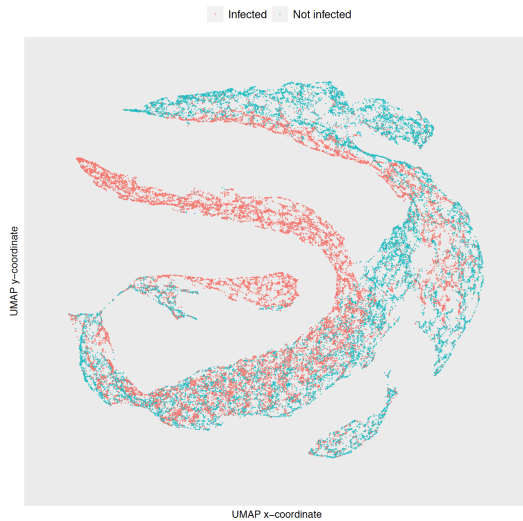
Figure 4 Analysis of the CMV-responding naive $V\delta2^{neg}\gamma\delta$ T cell proportion

A. Proportion of IFNγ-producing $V\delta2^{neg}\gamma\delta$ T cells was assessed by IFNγ ELISPOT in two CMV negative donors (Figure representative for one donor). $V\delta2^{neg}\gamma\delta$ T cells were purified with ARIA sorter and incubated with rIL18 either in media alone (simplicate), PMA 25ng/ml/1µg/ml ionomycine (simplicate) or non-infected or CMV-infected fibroblast (duplicates).

NI FSF, non infected fibroblats; CMV-inf FSF; CM-infected fibroblats

B. Cluster of CMV-responding naive $V\delta2^{neg}\gamma\delta$ T cells was assessed by UMAP analysis after RNA single cell sequencing of purified naive $V\delta2^{neg}\gamma\delta$ T cells either incubated with non-infected (green) or CMV-infected (red) fibroblasts. One experiment has been performed.
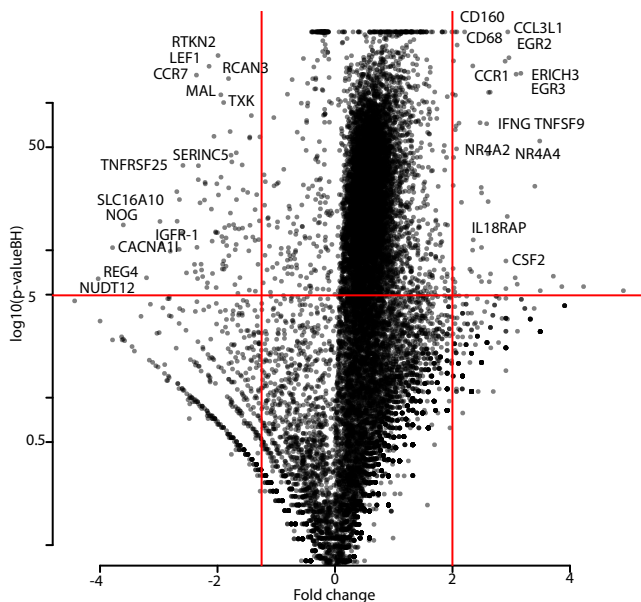
Figure 5

Figure 5 Analysis of the CMV-responding naive Vδ2^{neg} γδ T cell signature

UMAP analysis was performed on the entire cells and the entire genes sequenced (**Figure 4 B)**, allowing to observe a cluster (cluster 1) in which only γδ T cell from culture with CMV-infected fibroblasts were found together conversely to all others cells on the UMAP (put together as cluster 2). Gating separately cluster 1 and cluster 2 with Single-cell-virtual-master online software was performed and a volcano-plot allowed to observe the differential gene expression of cluster one compared to cluster 2. Those results are obtained form single cell analysis of one experiment.

Figure 6

A

B
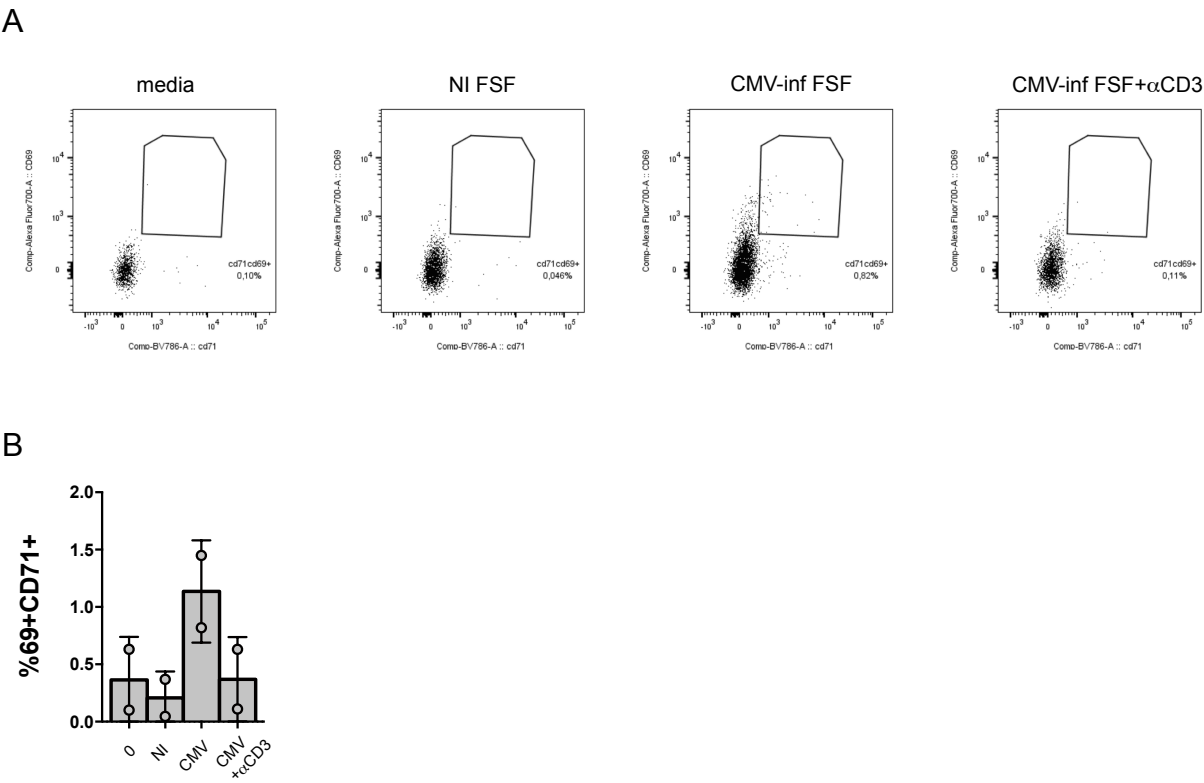
Figure 6 TCR involvement of naïve Vδ2^{neg} γδ T cell for their reactivity against CMV-infected cells.

PBMC from CMV negative donors (n=2) were negatively sorted with magnetic bead to purify Vδ2^{neg} γδ T cells. Then, cells were incubated with rIL18 in medium alone or with non-infected or CMV-infected fibroblast for 24 hours at 37°C. Moreover, for TCR-blocking, anti-CD3-V450 was incubated with cells for 15 minutes in PBS at 4°C, washed and incubated with rIL18 and CMV-infected cells. Then, cells were stained in order to gate CD45RA+CD27+ Vδ2^{neg} γδ T cells and analyze their CD69 and CD71 expression by flow cytometry. Example of CD69/CD71 gating of CD45RA+CD27+ Vδ2^{neg} γδ T cells in one donor (A) and histograms for two donors (B).

Supplemental Figure 1
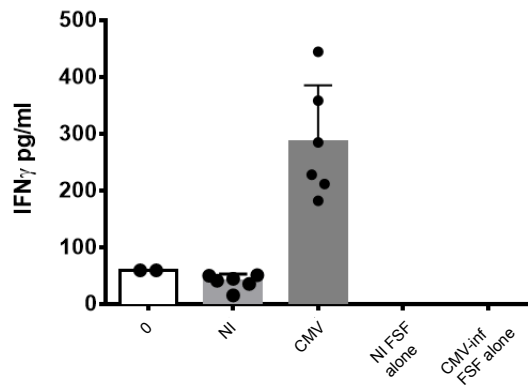
γδ T lymphocytes from a CMV negative healthy donor were negatively sorted with magnetic beads.

Then 50 000 cells per 96 wells-plate were incubated with rIL18 50ng/ml in media alone (duplicate)

and with non-infected and CMV-infected fibroblast (six wells for each conditions) overnight at

37°C. Then ELISA IFNγ was performed. NI, non-infected; FSF, foreskin fibroblats

DRAFT 2. Characteristics and time course of CMV infections leading to antiviral drug resistance

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Running title: Clinical time course of antiviral drug resistance

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Abstract

Introduction: CMV guidelines recommend testing for an antiviral drug resistance in kidney transplant recipients (KTR) who exhibit the two following criteria: 1/ a cumulative (Val)ganciclovir ((V)GCV) exposure >6 weeks and 2/ a treatment failure after >2 weeks of ongoing full dose (V)GCV. The objectives of this study were to describe the characteristics, the risk factors and the time course of CMV infections leading to antiviral drug resistance.

Material and methods: We performed a retrospective cohort study in 313 KTR treated for a first episode of CMV infection or disease from Oct 2004 to December 2017. Logistic and Cox regression analyses were performed for identifying risk factors of persistent CMV DNAemia, CMV recurrence, and antiviral drug resistance.

Results: Twenty (6%) of the 313 KTR developed an antiviral drug resistance. We found that persistent CMV DNAemia (HR = 6.25, CI95% 2.45-15.96, p <0.001) and clinical recurrence (HR = 7.20, CI95% 2.57-18.09, p <0.001) were the only risk factors associated with the development of antiviral drug resistance. These events are two major situations associated with viral replication during prolonged exposure to antivirals. Fifty-eight (18%) of the 313 KTR developed persistent CMV DNAemia and 65 (21%) clinical recurrence. The risk factors of persistent CMV DNAemia and clinical recurrence were similar: treatment with corticosteroid and anti-lymphocyte globulin, D+R- CMV serostatus, lymphopenia, high viral load and CMV disease.

Conclusion: We identified a new scenario leading to resistance to antivirals associating persistent CMV DNAemia and clinical recurrence. This perspective involving a chronological analysis of the history of the CMV episode could improve early interventions against antiviral drug resistance development.

Key words: cytomegalovirus, kidney transplantation, antiviral drug resistance

Introduction

Cytomegalovirus (CMV) infection and disease are important causes of morbidity and mortality in solid-organ transplant recipients (1). Based on the VICTOR study conducted in adult solid organ transplant recipients (SOTR) (2), oral Valganciclovir (VGCV) and intravenous Ganciclovir (GCV) are recommended in mild to moderate and in life-threatening CMV disease, respectively (3).

However, antiviral drug resistant CMV infection can occur and is associated with an increased morbidity and mortality (12,14,15). Antiviral drug resistant CMV infection is defined as a viral genetic alteration (on UL97 and/or UL54 gens) that decreases susceptibility to GCV and VGCV (4). It occurs in up to 10% of the SOTR with CMV infection (5-8). A strong immunosuppressive therapy, a lack of prior CMV immunity (Donor positive - recipient negative, D+R-), a high CMV load during CMV disease, an extended period of antiviral drug exposure and inadequate antiviral drug delivery are the most described risk factors of antiviral drug resistance (8-13).

In a recent case-control study, Fisher *et al.* pointed out that a longer prior exposure to GCV or VGCV was a major risk factor of antiviral drug resistance (FISHER 2017 cit 12). CMV guidelines recommend testing for an antiviral drug resistance in patients who exhibit a cumulative (V)GCV exposure >6 weeks, but this threshold relies on limited data (3). Moreover, it is unclear whether the cumulative exposure to GCV or VGCV must encompass prophylaxis and curative treatment. Indeed, antiviral drug resistant CMV infection can emerged either during universal prophylaxis (LIMAYE Lancet 2000), especially in patients receiving low dose VGCV (STEVENS Transplant Inf Dis 2015 : 17-163), or during persistent CMV DNAemia requiring prolonged treatment (COUZI AJT 2012; BOIVIN 2009). Solid organ transplant recipients can also be exposed to a prolonged (V)GCV therapy in case of CMV recurrence, but there is no evidence showing that CMV recurence is associated with antiviral drug resistance.

The objectives of this study were to determinate among the situations of prolonged (V)GCV exposure, i.e. an universal prophylaxis, a persistent CMV DNAemia requiring prolonged treatment or a CMV recurrence, which were associated with the development of antiviral drug resistance in a cohort of kidney transplant recipients (KTR).

Material and methods

Study design

We conducted a monocentric retrospective study at Bordeaux University Hospital, France, from October 2004 to December 2017 in order to identify those who underwent CMV infections or diseases requiring a CMV treatment. All were monitored for two years after their first treated CMV episode. This study was approved by the Institutional Review Board of the Bordeaux University Hospital.

Immunosuppression

HLA-sensitized patients were given rATG (THYMOGLOBULIN®, Sanofi-Aventis) protocol on day 0 (1.5 mg per kilogram of body weight) and on days 1 through 4 (1mg/kg). In non-HLA-sensitized patients, anti-IL2RA (20 mg) (SIMULECT®, Novartis Pharma SAS) was similarly used on day 0 and day 4. Maintenance immunosuppressive regimen was based on calcineurin inhibitors. Either tacrolimus (trough level 8-12 ng/ml for the first 3 months, then 5-10 ng/ml), or cyclosporine A (trough level 150-200 ng/ml for the first 3 months, then 75-125 ng/ml) were used. Mycophenolic acid was mainly used in association with calcineurin inhibitors. Steroids were used concomitantly, decreased in the first year and stopped only in non-HLA-sensitized. All acute rejections, which included both antibody-mediated and T cell–mediated acute rejections, were biopsy-proven. Glomerular filtration rate was expressed based on MDRD formula.

CMV prevention

CMV preventive strategy evolved over time. From 2004 to November 30th, 2006, D+R- patients and R+ rATG-treated patients received universal prophylaxis (valganciclovir 900 mg once daily) for 3 months. A preemptive strategy was used on anti-IL2 RA-treated patients. From December 2006 to June 2010, all patients were preemptively followed: CMV whole-blood real-time quantitative acid nucleic testing (QNAT) was performed once a week for the first three months, twice a month from month 3 to 6, and then at month 8, 10 and 12.

Finally, from July 2010 to December 2017, patients received universal prophylaxis either for 6 months (D+ R-) or for 3 months (R+). After the end of prophylaxis, CMV QNAT was performed at month 4 and 6 (for patients having received prophylaxis for 3 months), 9 and 12 post-transplantation. For all patients after month 12, CMV QNAT was performed annually or when CMV disease was clinically suspected.

CMV treatment

Intravenous (IV) GCV (5 mg/kg twice daily) or oral VGCV (900 mg twice daily) were initiated for the treatment of CMV infection or disease in view to obtain CMV DNAemia eradication. The dose was carefully adjusted at each outpatient visit, following the manufacturer's recommendations, using the Cockcroft–Gault formula.

CMV monitoring

CMV IgG serology was performed (Enzygnost anti-CMV/IgM and IgG [Dade Behring, Marburg, Germany] and Access CMV IgG and IgM [Beckman Coulter, Brea, CA]), following the manufacturer's recommendations.

CMV QNAT techniques evolved overtime. From October 2004 to June 2012, CMV QNAT was performed with an in-house whole blood real-time polymerase chain reaction, as previously described (17,18). QNAT was reported in copies/ml, however, equivalence in IU/ml was retrospectively calculated by using the WHO International Standard for human Cytomegalovirus (hCMV), in order to homogenize all the results before performing the statistical analysis. The thresholds of CMV DNAemia quantification and detectability were both at 250 IU/ml. From June 2012 to December 2018, CMV QNAT was performed with a whole blood real-time polymerase chain reaction using LightMix® Kit human Cytomegalovirus (TIB MOLBIOL GmbH, Berlin, Germany). The thresholds of CMV DNAemia quantification and detectability were at 1000 IU/ml and 250 IU/ml, respectively. The laboratory is following the program of Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) from 2004.

Baseline viral load was defined as the first positive viral load of the CMV event. During the viral monitoring of CMV disease, the assay was performed once a week until two consecutives negative CMV QNATs occurred or during the first seven weeks, then once a month until month 3.

Anti-CMV drug resistance was investigated when a significant increase of CMV load ($>1 \log_{10}$ UI/mL) was observed during antiviral therapy with VGCV or GCV and was defined as the presence of resistance-associated mutations in the *UL97* and/or *UL54* genes at the French National Cytomegalovirus Reference Center (Limoges, France), as previously reported (19).

Definitions of CMV events

We defined CMV infection as a CMV DNAemia $\geqslant$ 1000 IU/ml (based on the upper threshold of quantification from June 2012 to December 2018) in asymptomatic patients. CMV disease was defined as CMV syndrome or CMV tissue-invasive disease, consistent with the American

Society of Transplantation and the CMV Drug Development Forum recommendations (20). "Post-prophylaxis" CMV disease was defined as the first episode of CMV disease occurring >3 months (100 days) after transplantation. Early-onset disease was defined as the first episode of CMV disease occurring <3 months (100 days) after transplantation (21). CMV DNAemia eradication was defined as CMV QNAT < 250 IU/mL (threshold of detectability of our assays), in accordance with the guidelines (3).

CMV clinical recurrence was defined as a second CMV disease occurring concomitantly to a CMV DNAemia recurrence or a CMV DNAemia increase (3).

Statistical analysis

Univariate logistic-regression analysis to evaluate risks factors of CMV DNAemia persistence was performed, then covariates with a p-value <0.25 were included in a multivariate logistic-regression analysis. Results were expressed as Odds Ratios (OR) with 95% confidence intervals (95%CI).

Univariate Cox regression analysis for CMV clinical recurrence and antiviral drug resistance on the whole cohort and among "CMV DNAemia persistence" group was performed. Then, covariates with a <0.25 p-value and "prolonged anti-viral therapy" for the analyses among "CMV persistence" group were included in a multivariate Cox regression analysis. Two different multivariate models were performed with either "Baseline lymphocyte counts" or "7 weeks lymphocyte counts" in case of a p-value<0.25 observed in the univariate analysis. Results were expressed as Hazard Ratios (HR) with 95% confidence intervals (95%CI).

The survival curve for "free survival from mutation" was estimated with Kaplan-Meier method compared with log-rank test.

Analyses were performed with conventional statistical methods using the GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA) and the RStudio statistical software (Version 1.1.423 – © 2009-2018 RStudio, Inc).

Results

Study population

Among the 1792 kidney transplantations performed at our institution from October 2004 to December 2017, we identified 313 KTR requiring antiviral curative therapy for a first episode of CMV infection or disease (17.5%). Their baseline characteristics are shown in Table 1. This cohort was composed by 143 (45.7%) D+R- and 154 (49.2%) R+ patients. CMV prevention was based on universal prophylaxis with VGCV in 148 (47.3%), or a preemptive strategy in 148 (47.3%) of patients.

There were 95 (30.4%) CMV infections and 218 (69.6%) CMV diseases, equally distributed between 142 (45.4%) early-onset and 171 (54.6%) post-prophylaxis CMV infection or disease. The median baseline viral load was of 10900 (IQR: 4100-78200) IU/mL.

CMV DNAemia persistence after 7 weeks of (Val)ganciclovir exposure

The first treatment was VGCV in 148 (47.3%) and GCV in 165 (52.7%) of patients (Table 1). The median time to viral eradication was 27 days (IQR: 17-45). The median treatment duration was 49 days (IQR: 32-61). Thirty-eight (12.1%) patients received a secondary prophylaxis with VGCV.

Based on the VICTOR trial, CMV DNAemia persistence was analyzed 7 weeks after the initiation of the treatment by VGCV or GCV . Among the 313 KTR, 58 (18.5%) had CMV DNAemia persistence after 7 weeks days of (V)GCV exposure. The median viral load at 7 weeks in these 58 patient was low at 999 (IQR: 999-999) IU/mL.

We next determined the risk factors associated with this outcome in univariate and then multivariate analyses. Only D+R- serostatus (HR=3.70 CI95% [1.61- 9.09] p=0.003), high baseline CMV viral load at baseline (HR = 2.46 CI95% [1.82-3.39], p=0.002), and a lower 49 days lymphocyte count (HR=2.94 CI95% [1.15-8.33], p=0.033,) were independently associated with CMV DNAemia persistence after 7 weeks days of (V)GCV exposure (Table 3).

CMV clinical recurrence

CMV clinical recurrence occurred in 65 (20.8%) patients and was observed more frequently in patients with CMV DNAemia persistence after 7 weeks of (V)GCV exposure than in those with with CMV DNAemia eradication at 7 weeks (34.5% and 17.6%, respectively, p=0.006) (Figure 1). The median times (IQR) to CMV clinical recurrence were 84 (63-106) days post-treatment. Risk factors of clinical recurrence were identified with univariate (supplemental table 2), then multivariate analyses. rATG (HR=2.25 CI95% [1.16-4.35] p=0.016), steroids use (HR= 2.31 CI95% [1.19-4.46] p=0.012), D+R- serostatus (HR=2.56 CI95% [1.01-3.57] p=0.002), lower day 0 lymphocyte count (HR=2.08 CI95% [1.16-3.70] p=0.013), early onset infection or disease (HR=1.82, CI95% [1.02-3.13], p=0.041), and CMV disease (HR=2.57 CI95% [1.09-6.56], p=0.031) were independently associated with CMV clinical recurrence (Table 4).

Antiviral drug resistant CMV infection

Among the 313 KTR, 20 (6%) patients developed an antiviral drug resistant CMV infection. The median time to the diagnosis of antiviral drug resistance was 112 day (IQR: 80-146).

Universal prophylaxis was not associated with the development of antiviral drug resistance (HR=1.61, CI95% 0.7-3.7, p=0.2). On the opposite, CMV DNAemia persistence after 7 weeks days of (V)GCV exposure was strongly associated with the development of antiviral drug

resistance (HR=9.00, CI95% 3.59-22.59, p<0.001). Antiviral drug resistance was diagnosed in 22.4% (13/58) of patients with a CMV DNAemia persistence after 7 weeks days of (V)GCV exposure versus 2.7% (7/255) of patients with CMV DNAemia eradication. Antiviral drug resistance diagnosis was always performed after 7 weeks of (V)GCV exposure for these 13 patients (median time: 110 days, IQR: 84-140). CMV clinical recurrence was also strongly associated with the development of antiviral drug resistance (HR=9.58, CI95% 3.68-24.9, p<0.001). Antiviral drug resistance was diagnosed in 21.5% (14/65) of patients with CMV clinical recurrence versus 2.4% (6/248) of patients without CMV clinical recurrence. Antiviral drug resistance was diagnosis before clinical recurrence in 1 patient, at the same time in 1 patient and after in 12 patients.

In the univariate analyses, other risk factors associated with antiviral drug resistance were D+R- patients, lymphopenia, CMV disease (Table 2). In the multivariate analysis, only CMV DNAemia persistence after 7 weeks days of (V)GCV exposure (HR= 6.25, CI95% 2.45-15.96, p<0.001) and clinical recurrence (HR= 7.20, CI95% 2.57-18.09, p<0.001) were independently associated with antiviral drug resistant CMV infection (Table 2). Patients cumulating both a CMV DNAemia persistence after 7 weeks days of (V)GCV exposure followed by a CMV clinical recurrence had the higher incidence of antiviral drug resistance (50%) (Figure 1 and 2).

Discussion

In this study, we took advantage of a large cohort of KTR who received GCV or VGCV for CMV infection or disease to describe the different steps and kinetic of factors involved in the genesis of CMV antiviral drug resistance. Our sequential approach led us to identify a clinical situation frequently encountered before the genetic diagnosis of CMV mutation, which associates CMV persistence followed by a clinical recurrence.

We aimed to describe the characteristics, the risk factors and the time course of CMV infections leading to antiviral drug resistant CMV Infection, which remained one of the most problematic issue of CMV infection. We observed a 6% rate of antiviral drug resistance. This is consistent with other cohort studies (5-7). Many previous studies identified the lack of CMV immunity (D+R- at transplantation or at the end of the treatment) (9,23), the burden of immunosuppression (21) and CMV disease with high viral loads (8,12) as strong risk factors of antiviral drug resistance. We then tested these potential contributors. As the D+R- serostatus, lymphopenia, and CMV disease were associated with either a persistent CMV DNAemia at 7 weeks under treatment or clinical recurrence, all these baseline characteristics previously identified as risk factors for CMV drug resistance were no longer associated in the multivariate analysis. Only persistent CMV DNAemia and clinical recurrence were still associated with antiviral drug resistance. Whereas persistent CMV replication with a prolonged antiviral drug exposure is a well-known CMV complication leading to CMV drug resistance (8,12,13), one of the most important findings of this study is the identification of clinical recurrence as new risk factor of antiviral drug resistance.

They are two situations with active viral replication during prolonged exposure of GCV or VGCV and this study helps to better understand the link between longer duration of ganciclovir and the emergence of antiviral drug resistance. In 2000, Emery and Griffiths, using mathematical prediction models that were subsequently validated by in vivo studies, showed that in the presence of ganciclovir, mutant CMV strains have a survival advantage, compared with wild-type CMV strains. During prolonged exposure to ganciclovir, these resistant mutant strains become the dominant population and can ultimately lead to virologic or clinical failure (EMERY 2000 cit 13). Universal prophylaxis was first suspected to be associated with development of antiviral drug resistance (LIMAYE 2000). Limaye *et al* observed that viral replication under prophylaxis can lead to mutation development (LIMAYE 2002 JID). Then,

several studies showed that the use of universal prophylaxis is not associated with antiviral drug resistance (Couzi AJT 2012). Based on our results, we clarified and proved that more than prolonged antiviral exposure, it is viral replication despite prolonged antiviral exposure (CMV DNAemia persistence and clinical recurrence) which is associated to antiviral drug resistant CMV Infection development.

During time course of treated CMV infections, one of the first issue encountered by physicians during the treatment of a first CMV episode is the persistence of CMV DNAemia despite appropriate treatment. We observed in this cohort that 18.5% of the patients still had a CMV DNAemia persistence after 49 days of GCV or VGCV. We demonstrate that naïve D+R-serostatus, a low lymphocyte count, and a high basal CMV viral load were strongly associated with this outcome. The management of CMV DNAemia persistence based on international recommendations remains highly controversial. On one hand, antiviral treatment should be continued until obtaining a viral clearance, but on the other hand a viral replication despite cumulative VGCV exposure can lead to CMV antiviral drug resistance (3,7,8). This last point was confirmed in this study, suggesting that CMV persistence at 49 days under treatment should be considered as a new relevant entity among CMV complications.

The other major complication following a first CMV episode requiring antiviral therapy is clinical recurrence. In the VICTOR study, the incidence of clinical recurrence was 15% during a 1-year follow-up (16). In our study, 21% of the patients displayed a clinical recurrence. We confirmed that naïve D+/R- patients were at high risk of recurrence, probably because of a delay in the development of a protective CMV-specific cell immunity favors CMV recurrence (23-25). The burden of immunosuppression, which was characterized by rATG and steroids use was also a seminal factor involved in clinical recurrence. It could have participated to induce lymphopenia, another risk factor of recurrence (26). In line with this, we confirmed that clinical recurrence occurred mainly in patients with CMV disease (27), and high viral load (28). Unlike

CMV DNAemia recurrence, clinical recurrence systematically requires the use of antiviral therapy, leading to prolonged exposure.

Our study has several strengths. It is a large population displaying a CMV infection, including life-threatening disease, a homogeneous treatment based on the VICTOR study, and a regular and long-term follow-up. This real-life cohort included only KTR, because of the observed discordances between the different organ transplant characteristics, and the current need for organ transplant type-specific recommendations (FISHER 2017). But is has also potential weaknesses. Due to the relatively uncommon occurrence of mutation, our study was retrospective to include the maximum cases. They occurred during a long-time period during which transplant practices changed. As a single-center study, caution should be used when generalizing these results to other transplant centers with differing immunosuppression or other transplant practices.

In conclusion, we identified a new clinical scenario leading to antiviral drug resistance which associates CMV persistence and clinical recurrence. This chronological analysis of the history of CMV infection clarify the relevant entities which led to development of CMV antiviral drug resistance. Future studies should focus on improving management of these situations at risk of viral replication under prolonged antiviral exposure.

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Table 1: Patients characteristics and CMV Outcomes of the first treated CMV episode stratified by CMV DNAemia persistence at 7 weeks post-treatment

Patients characteristics	Total cohort n=313	CMV DNAemia persistence n=58	CMV DNAemia eradication n=255
Age at CMV onset (median; IQR)	57 (48-65)	56 (50-68)	57 (48-64)
Male sex (%)	205 (65)	39 (67)	166 (65)
Previous transplant (%)	41 (13.1)	10 (17)	31 (12)
Nephropathy			
Glomerular (%)	79 (25)	16 (27.5)	63 (25)
Tubulo-interstitial (%)	90 (29)	17 (29)	73 (29)
Vascular (%)	35 (11)	11 (19)	24 (9)
Diabetes (%)	20 (6)	2 (3.5)	18 (7)
Nephrectomy (%)	5 (2)	0 (0)	5 (2)
Congenital (%)	31 (10)	6 (10.5)	25 (10)
Unknown (%)	53 (17)	6 (10.5)	47 (18)
Dialysis before transplantation (%)	262 (84)	52 (90)	210 (82)
Antithymocyte globulin therapy (%)	115 (36.7)	24 (41)	91 (36)
Immunosuppressive drugs			
Tacrolimus (%)	224 (71.6)	42 (72)	182 (71)
Ciclosporin (%)	79 (25.2)	13 (22)	66 (26)
Mycophenolate mofetil (%)	286 (91.4)	51(89)	235 (92)
Azathioprine (%)	14 (4.5)	5 (9)	9 (4)
Steroid (%)	212 (67.7)	39 (67)	173 (68)
mTOR Inhibitors (%)	8 (2.6)	1 (2)	7 (3)
Acute rejection before CMV			
TCMR (%)	28 (8.9)	4 (7)	24 (9)
ABMR (%)	10 (3.2)	1 (2)	9 (4)
Glomerular filtration rate (ml/min/1.73 m^2, median, IQR)	40 (30-55)	40 (26-52)	40 (30-55)
Baseline tacrolimus blood concentration (ng/ml, median, IQR)	8 (7-10)	8.3 (6.2-10))	8 (7-10)
Day 21 tacrolimus blood concentration (ng/ml, median, IQR)	8 (6.8-10.4)	7.3 (5.9-9.4)	8.8 (7-10.6)
7 weeks tacrolimus blood concentration (ng/ml, median, IQR)	8.5 (7-10)	8 (6.4-8.8)	8.5 (7-10)
Baseline ciclosporin blood concentration (ng/ml, median, IQR)	132 (106-165)	120 (104-144)	140 (109-165)
Day 21 ciclosporin blood concentration (ng/ml, median, IQR)	140 (110-170)	151 (132-215)	140 (110-170)

7 weeks ciclosporin blood concentration (ng/ml, median, IQR)	120 (100-150)	151 (104-167)	120 (100-150)
Baseline Lymphocyte count (G/l, median, IQR)	0.77 (0.38-1.15)	0.70 (0.23-1)	0.79 (0.42-1.17)
Day 21 Lymphocyte count (G/l, median, IQR)	0.7 (0.42-1.2)	0.54 (0.40-0.83)	0.76 (0.46-1.20)
7 weeks Lymphocyte count (G/l, median, IQR)	0.76 (0.42-1.1)	0.49 (0.27-0.95)	0.8 (0.50-1.17)
CMV characteristics			
Donor (D)/recipient (R) serostatus at transplantation			
D+ / R- (%)	143 (45.7)	37 (64)	106 (42)
D+ / R+ (%)	107 (34.2)	14 (24)	93 (36)
D- / R+ (%)	47 (15)	3 (5)	46 (18)
D- / R- (%)	4 (1.3)	0 (0)	4 (1.5)
Unknown	12 (3.8)	4 (7)	8 (3)
Preventive strategy			
Universal prophylaxis (%)	148 (47.3)	35 (60)	113 (44)
Preemptive strategy (%)	148 (47.3)	19 (33)	129 (51)
Unknown	17 (5.4)	4 (7)	13 (5)
Type of onset episode			
CMV Infection (%)	95 (30.4)	7 (12)	88 (34.5)
CMV Disease (%)	218 (69.6)	51 (88)	167 (65.5)
Time from transplant to CMV onset (day, median, IQR)	137 (45-249)	215 (85-305)	105 (44-237)
Early onset CMV (%)	142 (45.4)	14 (24)	128 (50.5)
Post-prophylaxis infection or disease (%)	171 (54.6)	44 (76)	127 (49.5)
Initial anti-CMV curative therapy			
Valganciclovir (%)	148 (47.3)	18 (31)	130 (51)
IV Ganciclovir (%)	165 (52.7)	40 (69)	125 (49)
Treatment duration (day, median, IQR)	49 (32-61)	61 (48-84)	47 (30-56)
Baseline CMV DNAemia (UI/mL, median, IQR)	10900 (4150-73850)	67200 (13600-686000)	8600 (3800-41800)
Day 21 CMV DNAemia (UI/mL, median, IQR)	0 (0-1300)	1400 (999-5900)	0 (0-999)
7 weeks CMV DNAemia (UI/mL, median, IQR)	0 (0-0)	999 (999-999)	0 (0-0)
Time to viral eradication (day, median, IQR)	27 (17-44)	140 (65-285)	23 (15-33)
Received Secondary Prophylaxis (%)	38 (12.1)	4 (6.9)	34 (13.3)
CMV DNAemia recurrence	134 (43%)	11 (19%)	123 (48,2%)
CMV DNAemia increase		25 (43%)	
CMV clinical recurrence at 24 months	65 (21%)	20 (34,5%)	45 (17,6%)

Time to CMV clinical recurrence (day, median, IQR)	84 (63-106)	94 (76-138)	78 (62-97)
Antiviral drug resistance at 24 months	20 (6%)	13 (22,4%)	7 (2,7%)
Time to diagnosis of antiviral drug resistance (day, median, IQR)	112 (80-146)	110 (84-140)	115 (77-146)

Abbreviations: Antibody Mediated Rejection (ABMR); Cytomegalovirus (CMV); Donor (D); Interquartile (IQR); Recipient (R); T Cell Mediation Rejection (TCMR).

Table 2: Significant factors associated with CMV DNAemia persistence at 7 weeks post-treatment: multivariate analysis

Variable	Adjusted HR (95% CI)	p-value
D+/R- (vs Recipient CMV seropositive)	3.70 (1.61- 9.09)	0.003
Baseline CMV DNAemia	1.0000005 (1.0000002-1.000001)	0.002
7 weeks low lymphocyte count	2.94 (1.15-8.33)	0.033

Abbreviations: Cytomegalovirus (CMV); Donor (D); Recipient (R); 95% confidence intervale (95%CI).

Table 3: Significant factors associated with clinical recurrence: multivariate analysis

Variable	Adjusted HR (95% CI)	p-value
Antilymphocyte therapy used	2.25 (1.16-4.35)	0.016
Steroids used	2.31 (1.19-4.46)	0.012
D+/R- (vs Recipient CMV seropositive)	2.56 (1.01-3.57)	0.002
Early onset infection or disease	1.82 (1.02-3.13)	0.041
CMV Disease	2.57 (1.09-6.56)	0.031
Baseline low lymphocyte count	2.08 (1.16-3.70)	0.013

Abbreviations: Cytomegalovirus (CMV); Donor (D); Recipient (R); 95% confidence intervale (95%CI).

Table 4: Factors associated with Antiviral drug resistant CMV infection

	Univariate analysis		Multivariate analysis	
Variable	**Unadjusted HR (95% CI)**	**p-value**	**Adjusted HR (95% CI)**	**p-value**
Age at CMV onset	1.008 (0.97-1.04)	0.655		
Male sex	**0.51 (0.21-1.22)**	**0.133**		
Previous transplant	1.17 (0.34-3.99)	0.8		
Dialysis before transplantation	0.72 (0.24-2.16)	0.562		
ATG	1.64 (0.68-3.93)	0.27		
Steroids	0.88 (0.35-2.21)	0.79		
Tacrolimus	**3.69 (0.86-15.90)**	**0.079**		
Ciclosporin	**0.032 (0.07-1.38)**	**0.126**		
Mycophenolate mofetil	0.83 (0.19-3.58)	0.805		
Azathioprine	**2.36 (0.55-10.18**	**0.249**		
eGFR	0.99 (0.97-1.02)	0.778		
TCMR	**4.54 (0.6-34.48)**	**0.142**		
D+/R-	**4.54 (1.54-13.51)**	**0.006**		
Universal prophylaxis	1.61 (0.7-3.7)	0.257		
Early onset infection or disease	**1.82 (0.75-4.54)**	**0.19**		
CMV Disease	**8.46 (1.13-63.19)**	**0.037**		
Baseline CMV DNAemia	1 (1-1)	0.919		
Day 21 CMV DNAemia	1 (1-1)	0.618		
Baseline low lymphocyte count	**3.12 (1.07-9.09)**	**0.035**		

7 weeks low lymphocyte count	**7.69 (1.7-33.3)**	**0.0079**		
CMV DNAemia persistence	**9.00 (3.59-22.59)**	**<0.001**	6.25 (2.45-15.96)	<0.001
Clinical recurrence	**9.58 (3.68-24.9)**	**<0.001**	7.20 (2.57-18.09)	<0.001

Abbreviations: Antithymocyte Globulin (ATG); Cytomegalovirus (CMV); Donor (D); estimated Glomerular Filtration Rate (eGFR); Recipient (R); T Cell Mediation Rejection (TCMR).

Multivariate analysis was performed for all covariates in case of a p-value<0.25 observed in the univariate analysis (in bold). We decided, for clarity, to show only significant factors in multivariate analysis.

Supplemental

Supplemental 1: Factors associated with CMV DNAemia persistence at 7 weeks post-treatment, univariate and multivariate analysis

Variable	Unadjusted HR (95% CI)	p-value	Adjusted HR (95% CI)	p-value
Age at CMV onset	1.003 (0.98-1.02)	0.770		
Male sex	1.1 (0.61-2.05)			
Previous transplant	1.51 (0.66-3.18)	0.303		
Dialysis before transplantation	**1.73 (0.75-4.73)**	**0.235**		
ATG	1.36 (0.75-2.47)	0.309		
Steroids	1.03 (0.55-1.85)	0.929		
Tacrolimus	1.05 (0.57-2.04)	0.874		
Ciclosporin	0.99 (0.41-1.59)	0.398		
Mycophenolate mofetil	0.62 (0.26-1.65)	0.304		
mTOR inhibitors	0.54 (0.03-3.04)	0.567		
Azathioprine	**2.58 (0.77-7.78)**	**0.101**		
Baseline ciclosporin blood concentration	1.01 (0.99-1.03)	0.398		
Day 21 ciclosporin blood concentration	1.01 (0.99-1.02)	0.732		
7 weeks ciclosporin blood concentration	1.01 (0.99-1.02)	0.784		
Baseline tacrolimus blood concentration	1.02 (0.88-1.19)	0.798		

Day 21 tacrolimus blood concentration	**1.11 (0.99-1.27)**	**0.087**		
7 weeks tacrolimus blood concentration	**1.20 (1.02-1.43)**	**0.0347**		
eGFR	1.01 (0.99-1.02)	0.521		
TCMR	**2.33 (1.02-6.25)**	**0.064**		
ABMR	2.08 (0.38-33.3)	0.49		
D+/R-	**2.86 (1.59-5.55)**	**<0.001**	**3.70 (1.61-9.09)**	**0.003**
Preemptive strategy	**2.19 (1.22-4.06)**	**0.010**		
Post-prophylaxis infection or disease	**3.17 (1.69-6.25)**	**<0.001**		
CMV Disease	**1.78 (1.25-3.33)**	**0.001**		
Baseline CMV DNAemia	**1.0000003 (1.0000001-1.0000006)**	**0.011**	**1.0000005 (1.0000002-1.000001)**	**0.002**
Day 21 CMV DNAemia	**1.0000067 (0.9999991-1.0000187)**	**0.129**		
Baseline Lymphocyte count	**1.55 (0.89-3.03)**	**0.16**		
7 weeks Lymphocyte count	**3.13 (1.41-7.69)**	**0.007**	**2.94 (1.15-8.33)**	**0.033**

Abbreviations: Antibody Mediated Rejection (ABMR); Antithymocyte Globulin (ATG); Cytomegalovirus (CMV); Donor (D); estimated Glomerular Filtration Rate (eGFR); Recipient (R); T Cell Mediation Rejection (TCMR).

Multivariate analysis was performed for all covariates in case of a p-value<0.25 observed in the univariate analysis (in bold). We decided, for clarity, to show only significant factors in multivariate analysis.

Supplemental 2: Factors associated with clinical recurrence, univariate and multivariate analysis

Variable	Unadjusted HR (95% CI)	p-value	Adjusted HR (95% CI)	p-value
Age at CMV onset	1.01 (0.99-1.02)	0.847		
Male sex	1.22 (0.74-2)	0.447		
Previous transplant	1.35 (0.70-2.58)	0.365		
Dialysis before transplantation	1.14 (0.59-2.15)	0.72		
ATG	**1.45 (0.85-2.44)**	**0.168**	**2.27 (1.16-4.35)**	**0.016**
Steroids	**1.41 (0.81-2.44)**	**0.227**	**2.31 (1.19-4.47)**	**0.012**
Tacrolimus	1.24 (0.70-2.18)	0.455		
Ciclosporin	1.14 (0.63-2)	0.686		
Mycophenolate mofetil	1.08 (0.43-2.69)	0.866		
mTOR inhibitors	2.08 (0.29-14.9)	0.469		
Azathioprine	**2.02 (0.81-5.03)**	**0.131**		
eGFR	1.003 (0.99-1.02)	0.686		
TCMR	1.39 (0.78-2.48)	0.261		
D+/R-	**2.49 (1.49-4.16)**	**<0.001**	**2.56 (1.37-4.76)**	**0.002**
Preemptive strategy	0.93 (0.59-1.49)	0.777		
Early onset infection or disease	**1.39 (0.85-2.27)**	**0.188**	**1.82 (1.02-3.23)**	**0.041**
CMV Disease	**2.97 (1.47-6.01)**	**0.002**	**2.57 (1.09-6.06)**	**0.031**
Baseline CMV DNAemia	1 (1-1)	0.975		
Day 21 CMV DNAemia	1 (1-1)	0.646		
Baseline Lymphocyte count	**0.61 (0.38-1)**	**0.05**	**2.08 (1.16-3.75)**	**0.013**

Abbreviations: Antibody Mediated Rejection (ABMR); Antithymocyte Globulin (ATG); Cytomegalovirus (CMV); Donor (D); estimated Glomerular Filtration Rate (eGFR); Recipient (R); T Cell Mediation Rejection (TCMR).

Multivariate analysis was performed for all covariates in case of a p-value<0.25 observed in the univariate analysis (in bold). We decided, for clarity, to show only significant factors in multivariate analysis.
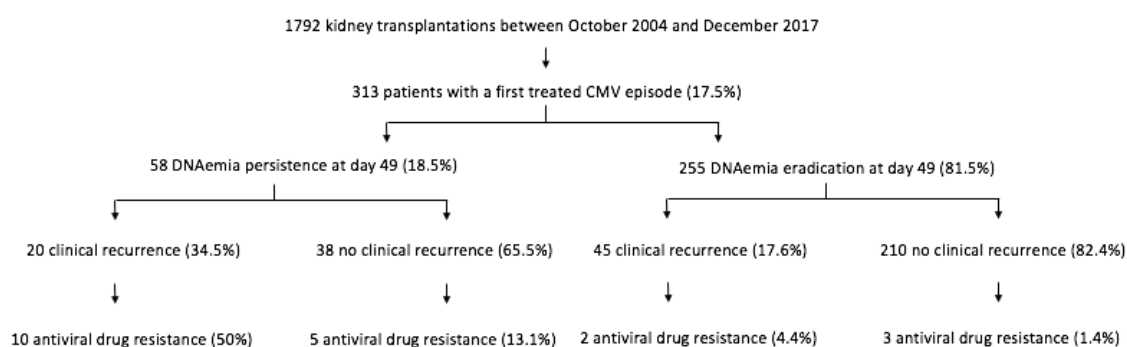
Figures

Figure 1: Flowchart of the study. Description of the time course and the different issues of CMV episodes requiring antiviral therapy, leading to the development of an antiviral drug resistant CMV infection.

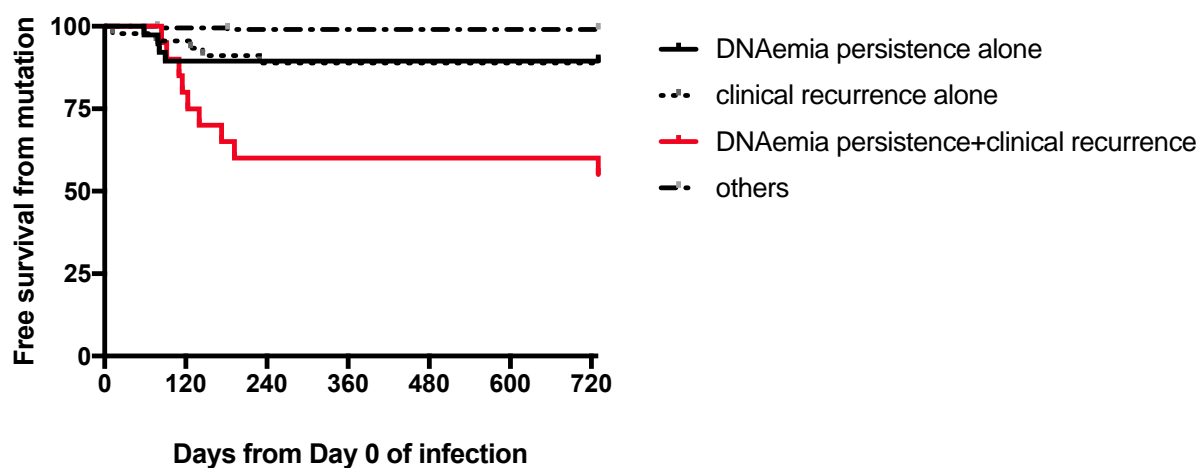**Figure 2**: Kaplan Meier curve estimates of Mutation-free survival stratified on DNAemia persistence at week 7, clinical recurrence, both of them, or neither of them.

DNAemia persistence at week 7 followed by clinical recurrence is strongly associated with antiviral drug resistance.
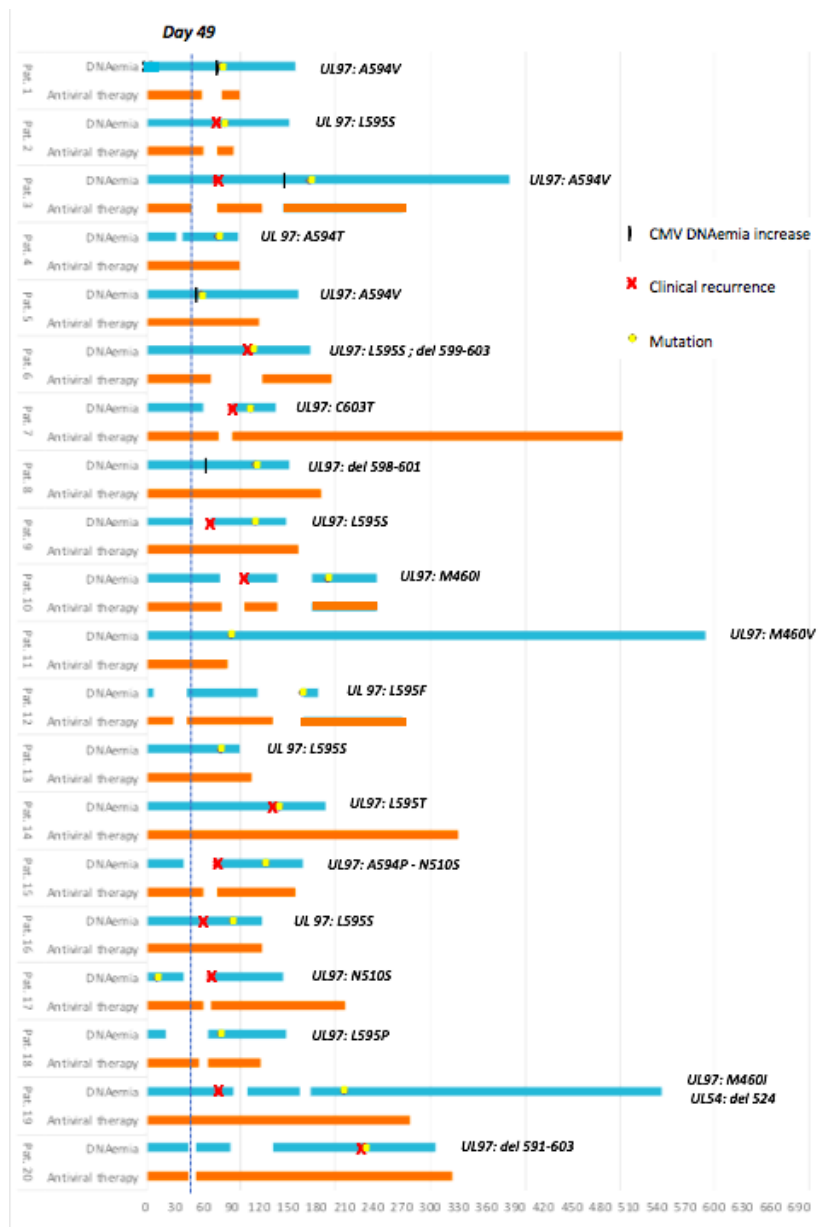
Figure 3: Description of the CMV infection history of the twenty patients who developed an antiviral drug resistance CMV infection.

DRAFT3. Prevention of Cytomegalovirus Disease by Everolimus in Seropositive Kidney Transplant Recipients

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Abstract

Background: The use of everolimus (EVR) was shown to decrease cytomegalovirus (CMV) infection in CMV seropositive kidney transplant recipients. In this multicenter trial EVERCMV, we tested if EVR could be a new preventive strategy in these patients.

Methods: We randomized 186 CMV seropositive kidney transplant recipients to receive EVR (n=95) or mycophenolate (MPS, n=91) in association with basiliximab, cyclosporin, and prednisone. No universal prophylaxis was given. The composite primary endpoint was CMV DNAemia, CMV treatment, graft loss, death, and discontinuation of the study at 6 months post-transplantation.

Findings: 48.3% and 80.5% of patients in the EVR and MPS groups reached the primary endpoint, respectively (p<0.0001). Patients receiving EVR showed a lower incidence of CMV DNAemia (39.2% vs. 77.8%, p<0.0001), however EVR was stopped in 35.6% of them. Only 21.4% of patients with ongoing EVR treatment experienced CMV DNAemia (HR=0.14, CI95% 0.08-0.24, p<0.0001). Only 7.4% of them required a CMV treatment because CMV viral loads were low (maximal: 5846 IU/mL), when compared to 36.4% in those with EVR discontinuation and 46% in the MPA group (HR=0.08, CI95% 0.03-0.2, p<0.0001). The incidences of rejection, adverse events and graft loss were similar in the EVR and MPS groups.

Interpretation and Findings: EVR was associated with a reduced incidence of CMV DNAemia. Among them, a minority required a treatment. EVR could be considered as a new CMV preventive strategy in CMV seropositive recipients as long as it is tolerated and continued.

Introduction

Each year, around 150 000 solid-organ transplantations are performed worldwide. In Europe and US, around 25 % of kidney transplant recipients (KTR) are CMV seronegative and receive a kidney from a seropositive donor (D+R-), while 60% are CMV seropositive (R+) at the time of transplantation {Zuhair:2019hv}. These two populations are at high risk of developing CMV disease with an incidence reaching 15 to 20% in D+R- and 5 to 10% in R+ KTR {Humar:2010fw} {Witzke:2012kt}. CMV disease is associated with a significant morbidity, and indirect effects such as an increased incidence of rejection and lower graft and patient survivals {Kotton:2013cz}.

Prevention of CMV disease relies on the so-called universal prophylaxis or a preemptive strategy {Kotton:2018ir}. Universal prophylaxis consists of giving antiviral treatment to all patients, whereas the preemptive strategy is based on regular screening for CMV infection in order to initiate treatment only in recipients who develop an asymptomatic infection. However, both of them have important drawbacks {Florescu:2014bs}. Universal prophylaxis is associated with severe post-prophylaxis CMV disease and frequent valganciclovir side effects {Luan:2011ee}. Preemptive strategy is very difficult to implement and is associated with severe early-onset CMV disease {Kaminski:2016bu}. Therefore, there is an unmet need for new strategies for preventing CMV disease in KTR.

Over the past years, a growing body of evidence has shown that the use of mTOR inhibitors (mTORi) had anti-CMV properties. *In vitro*, mTORi could directly inhibit CMV replication in macrophages {Poglitsch:2012bl}, deviate the CMV-mediated immune evasion by blocking mTORC1 activity in myeloid cells, and potentiate the generation of CMV-specific memory T-cell {Gerna:2011gs}{Nashan:2012ca}{Havenith:2013is}{Bak:2018gp}. In large randomized trials, a reduction of post-transplant CMV events was reported in D+R- and R+ KTR receiving mTORi, either in association with mycophenolate acid (MPA) {Mallat:2017fk}, or with

reduced dose of calcineurin inhibitors {Cibrik:2013gr} {Brennan:2011ep} {Qazi:2017gf} {Sommerer:2019ev} {Berger:2019cn}. However, in these large studies CMV was not the primary endpoint, the absence of a routine monitoring of CMV QNAT could lead to underestimate the incidence of CMV infection, and there was no standard use of prophylaxis or preemptive therapy. Three monocentric trials with CMV infection as primary endpoint also showed a reduction of CMV infection or disease in R+ patients included in everolimus (EVR) arms {Ferreira:2019hq,deSandesFreitas:2018fd, TedescoSilva:2015ev}. However, CMV infection was mainly diagnosed based on pp65 antigenemia, which is less sensitive {Kotton:2018ir}.

Based on these studies, the 2018 Consensus Guidelines on the Management of Cytomegalovirus in Solid-organ Transplantation proposed that the use of EVR could be a potential approach to decrease CMV infection and disease only in R+ KTR {Kotton:2018ir}. As 30% of patients discontinue mTORi due to side effects {SilvaJr:2010jx, Pascual:2018kq}, the incidence of significant CMV DNAemia or CMV disease in patients with ongoing EVR treatment could be even much lower. EVR could thus be a new preventive strategy as long as R+ KTR are receiving this drug. In order to address this question, we conducted the EVERCMV study, which was a multicentric, randomized, open-label, parallel group study in CMV R+ KTR, comparing EVR vs. MPA, including both an intention-to-treat and on-treatment analyses, with CMV DNAemia and CMV DNAemia requiring treatment as endpoints.

Material and methods

Study design

This was a multicentric, randomized, 12-month open-label trial aiming to compare the incidence of CMV DNAemia in kidney transplant recipients receiving EVR or MPA and no CMV universal prophylaxis by valganciclovir. Patients were included in 9 French transplant

units (Bordeaux, Toulouse, Lyon, Caen, Strasbourg, Necker, Limoges, Brest, Kremlin-Bicetre). The protocol was approved by an independent ethics committee (CPP number: 2013/57) and registered in the ClinicalTrials.gov database (NCT02328963). All subjects signed a written informed consent before enrollment and the study was conducted according to good clinical practices and the Declaration of Helsinki. Novartis funded this study.

Population

CMV seropositive adult recipients of ABO-compatible renal transplants from living or deceased donors were considered for enrollment. We excluded patients receiving or having previously received an organ transplant other than a kidney or receiving a graft from a non-heart-beating donor. We also excluded patients with calculated panel reactive antibody > 85%, the presence of historical or current anti-HLA donor-specific antibodies by Luminex assays, a positive cytotoxic crossmatch, and total ischemia over 36 hours. Women should have a negative serum pregnancy test at enrolment and to agree in using approved contraceptives methods during the duration of the study.

Randomization and treatments

Participants fulfilling eligibility criteria included before the transplant surgery and randomized to one of the two immunosuppressive regimens via a centralized procedure. Randomization was performed with a 1:1 allocation, permuted blocks of size 6 and 8, and stratified according to CMV serology of the donor (positive or negative), and the transplant center (Bordeaux vs. other centers).

All the patients received basiliximab induction (20 mg) on days 0 and 4 and, methylprednisolone 500 mg at day 0, 120 mg at day 1, and then prednisone 20 mg/day from day 3. Corticosteroid dosing was tapered according to local standard practice but to not less

than 5 mg per day for the duration of the study. All patients received trimethoprim-sulfamethoxazole for pneumocystis prophylaxis.

In the EVR group, patients received cyclosporin twice daily, adjusted to maintain whole blood concentrations between 100 and 200 ng/mL from day 0 to month 2, 75 to 150 ng/mL from month 2 to month 4, 50 to 100 ng/mL from month 4 to month 6, and 25 to 50 ng/mL from month 6 to 12, based on the A2309 study {SilvaJr:2010jx}, and EVR 1.5 mg twice daily, adjusted to maintain whole blood trough concentrations between 5 and 8 ng/ml.

In the MPA group, patients received cyclosporin twice a day, adjusted to maintain whole blood trough concentrations between 150 and 200 ng/mL from day 0 to month 3, than 100 to 150 ng/mL from month 3 to month 12 and MPA 1080 mg twice daily for one month, then 720 mg twice daily {Budde:2011hx}.

CMV preemptive strategy

No pharmacological prophylaxis for CMV infection was used. Real time whole blood CMV QNAT was performed every week during the first three months, then every two weeks from month 3 to month 4, then at months 5 and 6. CMV QNAT was performed with a real-time polymerase chain reaction in whole blood in all centers. The various CMV QNAT used in all the center were calibrated with the World Health Organization International Standard for CMV, in order to homogenize all the results reported as IU/ml. All the laboratories are following the program of Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) from 2004. Patients who developed a whole blood CMV DNAemia or CMV disease received either IV ganciclovir or valganciclovir according to local standard practice.

Endpoints and definitions

The primary endpoint was defined as the composite of CMV DNAemia, CMV treatment, graft loss, death, and discontinuation of the study at 6 months post-transplantation. CMV DNAemia was defined as a single positive whole blood CMV QNAT.

Secondary endpoints included the proportion of patients with CMV DNAemia requiring treatment, CMV disease defined as CMV syndrome or CMV tissue-invasive disease, consistent with the American Society of Transplantation and the CMV Drug Development Forum recommendations {Ljungman:2017ki}, the occurrence of CMV mutation (*UL97* or *UL54*) associated with a resistance to an anti-CMV therapy, the proportion of patients with CMV treatment failure defined as the absence of viral eradication at day 49 after the initiation of anti-CMV therapy based on the VICTOR study {Asberg:2009jp}, the proportion of BK virus DNAemia at month 1, 3, 6 and 12, the proportion of patients with acute rejection which were graded according to the Banff 2013 classification on for-cause biopsies {Haas:2014du}, the incidence of *de novo* DSA analyzed on a Luminex platform, estimated glomerular filtration rate (eGFR by MDRD formula), and spot urine protein/creatinine ratio (Up/c). Safety analysis included incidence of adverse events, delayed graft function which was defined as the need for dialysis during the first week after transplantation, infections, and malignancies.

CMV-specific cellular immunity

CMV-specific cellular immunity was analyzed with the QuantiFERON-CMV assay in patients included at the Bordeaux Hospital (n=83). It was available at day 7, day 14 and month 6 post transplantation, in 33, 32 and 24 patients with ongoing EVR treatment and 41, 43 and 39 patients with ongoing MPA treatment, respectively. QuantiFERON-CMV (n°0350-0201, qiagen) was performed as previously described {Manuel:2013ht} and read with QUANTA-Lyser® 2 Inova Diagnostics. Results were analyzed with CMV v3.03 software.

Sample size

The sample size was calculated to demonstrate a minimum decrease of 50% of the incidence of CMV DNAemia from 40 % in the MPA group to 20% in the EVR group in the absence of universal prophylaxis. The A2309 study in 2011 showed that both strategies resulted in a similar rate of death, graft loss or loss of follow-up (10%). Therefore, the proportion of expected efficacy failure was 46% in the MPA arm (10% + 40% * 90%) and 28% (10% + 20% * 90%) in the EVR arm. The formula used was based on the normal approximation of the binomial distribution. Assuming a minimum power of 80% and a type I error of 5%, 113 patients were planned to be included in each group for a total of 226 patients.

Statistical analysis

A statistical analysis plan was developed and validated by the Trial Scientific Committee before database lock and analyses. The primary efficacy analyses were conducted on the intent-to-treat population (all randomized patients). The primary composite endpoint at 6 months was a comparison between the two groups using an exact Fischer test. Secondary analysis was a comparison between the two groups of the CMV infection occurrence using a Cox proportional hazard model adjusted for CMV serology of the donor (positive or negative), the transplant center (Bordeaux vs. other centers) and a time-dependent measure of the exposure to EVR to take into account an interruption of EVR or a switch to EVR. CMV disease cumulative rates were compared using an exact Fischer test. The 95% confidence intervals of adverse events proportion in each group are calculated using the exact binomial distribution. For the descriptive variables, Wilcoxon-Mann-Whitney or Student tests were used according to the distribution of the variables and exact Fischer test were used when appropriate. $p < 0.05$ was considered statistically significant. All statistical tests were performed with the software SAS 9.4 (SAS Institute, Cary, NC, USA).

Results

Patients' characteristics

Patients were enrolled in this trial between May 2014 and October 2017 and were followed for one year. We randomized 186 patients (95 in EVR group, 91 in MPA group), but only 174 were transplanted and analyzed (EVR group, n=87, and MPA group, n=87) (Figure 1). There was no difference in the number of patients completing the study (71 vs. 72 in the EVR and MPA group, respectively). Baseline demographic characteristics were well balanced between the two groups (table 1).

Table 1: Demographic characteristics of the transplant recipients

	EVR (n =87)	MPA (n =87)
Recipient		
Age (years), mean (SD)	63.0 (14.1)	61.5 (14.7)
Male, n (%)	58 (66.7)	62 (71.3)
Donor		
Age (years), mean (SD)	59.3 (15.2)	56.9 (18.2)
Male, n (%)	51 (58.6)	46 (52.9)
Living donor, n (%)	9 [a] (10.7)	3 [b] (3.6)
Expanded criteria deceased donor, n (%)	51 (58.6)	47 (54)
Cold ischaemia time (hours), mean (SD)	12.3[a] (6.2)	13.4[b] (5.9)
HLA sensitized, n (%)	8[c] (9.3)	10 (11.5)
HLA A mismatches, n (0/1/2)	7/39/41	13/41/33
HLA B mismatches, n (0/1/2)	3/32/52	6/29/52
HLA DR mismatches, n (0/1/2)	21/43/23	25/36/26
HLA DQ mismatches, n (0/1/2)	21/50/16	29/38/20
CMV Seropositive Donor, n (%)	47 (54)	48 (55.2)

At day 30, 90, 180, and 365, median cyclosporin whole blood concentrations were 177, 124, 100, and 91 ng/ml in the EVR group, and 194, 138, 113, and 130 ng/ml in the MPA group, respectively. Cyclosporin area under the curves from day 7 to day 365 (including 17 measurements of cyclosporin trough levels) was lower in the EVR group than in the MPA group (median [IQR]: 35413 [18313-44669] vs. 43752 [27832-52846], respectively, p=0.01). At day 30, 90, 180, and 365, median EVR whole blood concentrations were 6, 5, 5, and 5 ng/ml respectively.

Primary endpoint and CMV DNAemia occurrence

The rates of patients who reached the composite primary endpoint were 48.3% and 80.5% in the EVR and MPA groups, respectively (p<0.0001, Figure 2). EVR group had a lower risk of reaching the primary endpoint when adjusted on the investigation center and the donor CMV serology (OR CI95% 0.21 [0.11-0.43], p<0.0001). Patients of the EVR group showed a lower incidence of CMV DNAemia (39.2% vs. 77.8%, p<0.0001, Figure 2), and received less CMV treatment compared to those of the MPA group (21.8% vs. 47.1%, p=0.0007, Figure 2). Graft losses, deaths, and discontinuation of the study before month 6 were similar between the 2 groups at 8% vs. 9.2% (p=1.0), 3.4% vs. 1.1% (p=0.62), and 2.3% vs. 5.7% (p=0.44), respectively (Figure 2).

The cumulative incidence of CMV DNAemia is depicted in the Figure 3A. The average time to first positive CMV QNAT censored for competitive risks were 4.2 ± 2.3 months in the EVR and 2.3 ± 2.1 months in the MPA. The cumulative incidence of CMV DNAemia requiring antiviral treatment is depicted in Figure 3B. The average time to first positive CMV QNAT

requiring treatment censored for competitive risks were 4.6 months ± 2.1 months in the EVR group and 3.1 ± 2.3 months in the MPA group.

Severity of CMV infection

The median (IQR) of maximal CMV QNAT was 740 (499-4292) UI/ml in the EVR group and 2117 (499-7736) in the MPA group (p=0.29). Eleven patients developed a CMV syndrome and two a tissue-invasive CMV disease (colitis) during the first 12 months post-transplantation. CMV disease occurred in 5.3% (4/76) and 11.8% (9/76) of patients of the EVR and MPA groups, respectively (p=0.2). Among the patients who were treated for CMV DNAemia or disease, treatment failure at 8 weeks (defined as the persistence of CMV DNAemia) occurred in 6.7% and 35.3% of patients in the EVR and MPA groups, respectively (p=0.04) (table 2). No CMV mutation on *UL97* or *UL54* were found.

Table 2: Severity of CMV DNAemia or disease

	EVR (n =87)	MPA (n =87)	p
Maximal viral load (IU/mL), median (IQR)	740[a] (499-4292)	2117[b] (499-7736)	0.29
CMV disease or syndrome at month 12, n (%)	4/76 (5.3)	9 /76 (11.8)	0.24
Treatment failure[1] at 3 weeks, n (%)	1/14 (7.1)	13/37 (35.1)	0.08
Treatment failure[1] at 8 weeks, n (%)	1/15 (6.7)	12/34 (35.3)	0.04
CMV mutation (*UL97* or *UL54*), n	0	0	

[1] Treatment failure: persistence of CMV DNAemia

[a] n=31, [b] n=63

On-treatment analyses

EVR and MPA were stopped in 31 (35.6%) and 7 (8%) patients, respectively. EVR was discontinued in 23 patients (74.2%) because of adverse events and in 8 others based on the

decision of the investigators. It was replaced by MPA in 29 patients (72.5%). Consequently, we performed additional on-treatment analyses to determine the incidence of CMV DNAemia in patients with ongoing and discontinued EVR treatment.

Patients with ongoing EVR treatment experienced less CMV DNAemia (12/56; 21.4%), than patients with EVR discontinuation (19/31; 61.3%) or the MPA group (61/87; 70.1%) (Figure 3C). Using a time-dependent Cox-proportional hazard regression model, adjusted for the investigation center and CMV donor serology, patients with ongoing EVR treatment had a much lower risk of CMV DNAemia when compared other patients (HR 0.14, CI95% 0.08-0.24, p<0.0001).

Therefore, only 7.4% of patients with ongoing EVR treatment experienced CMV DNAemia requiring treatment, when compared to 36.4% of patients with EVR discontinuation, and 46% of patients of the MPA group (Figure 3D). Using a time-dependent Cox-proportional hazard regression model, patients with ongoing EVR treatment had a very low risk of CMV DNAemia requiring antiviral drug (HR 0.08, CI95% 0.03-0.2, p<0.001).

Lastly at month-12, only 4.1% (2/49) of patients with ongoing EVR treatment experienced CMV disease, when compared to 7.4% of patients with EVR discontinuation (2/27), and 11.8% of patients in the MPA group (9/76) (p=0.33) (Figure 3E). Importantly, the maximal CMV QNAT observed in patients with ongoing EVR treatment was 5846 IU/mL, when compared to 173 300 IU/mL in patients with EVR discontinuation, and 219 520 IU/mL in the MPA group (Figure 3F).

CMV-specific cellular immunity

Patients with ongoing EVR treatment had higher levels of QuantiFERON-CMV at day 7 (p=0.009), day 14 (p=0.002) and month 6 (p=0.009), when compared to those with ongoing MPA treatment (Figure 4). Levels of QuantiFERON-CMV increased between day 7 and day

14 post transplantation (p=0.038), and then before CMV events in patients with ongoing EVR treatment. By contrast in patients with ongoing MPA treatment, the increase in QuantiFERON-CMV levels was observed later at month 6 (p=0.027, when compared to day 7).

Rejection, graft survival and adverse events

The incidences of *de novo* DSA at 12-months post-transplantation were 6.9% and 10.3% (p=0.6) in the EVR and the MPA groups, respectively (Table 3). The incidence of 12-month biopsy-proven T-cell and antibody-mediated acute rejection was at 16.1% and 17.2% in the EVR and the MPA groups, respectively. Mean [SD] eGFR was not statistically different between the EVR and the MPA groups at 12-month post-transplantation (40.7 [15.5] vs. 46.7 [21.9] ml/min/1.73m2, p=0.06). The 12-month median U p/c ratio was low in the two groups (30 and 16 mg/mmol, respectively). One-year graft loss occurred in 10.3% and 10.3% of the patients, respectively.

At month-12, we did not observe any difference between the EVR and MPA groups in the incidence of adverse events, serious adverse events, wound-healing delay, delayed graft function, lymphocele, infections, BK viremia, anemia, leucopenia, platelet counts, mean, HDL and LDL cholesterol. Only triglyceride level was higher in the EVR arm at month-12 (Table 3).

Table 3: Rejection, graft survival and adverse events

	EVR (N =87)	MPA (N =87)
De novo DSA at month 3, n (%)	3 (3.4)	2 (2.3)
median sum of DSA MFI (IQR25-75) at month 3	1393[a] (830-2000)	993[b] (853-1133)
De novo DSA at month 12, n (%)	6 (6.9)	9 (10.3)
median sum of DSA MFI (IQR25-75) at month 12	8837[c] (1129-13236)	2579[d] (0-5849)

Borderline, n (%)	7 (8.0)	9 (10.3)
Acute T-cell mediated rejection, n (%)	10 (11.5)	14 (16.1)
Active antibody-mediated rejection, n (%)	4 (4.6)	1 (1.1)
eGFR at month 6 (ml/min/1.73m^2), mean (SD)	40.8[e] (14.8)	44.4[f] (23.6)
eGFR at month 12 (ml/min/1.73m^2), mean (SD)	40.7[g] (15.5)	46.7[h] (21.9)
6-month proteinuria/creatininuria (mg/mmol), median (IQR25-75)	29[i] (17-78)	20[j] (11-33)
12-month proteinuria/creatininuria (mg/mmol), median (IQR25-75)	30[k] (16-46)	16[l] (10-36)
Graft loss at month 12, n (%)	9 (10.3)	9 (10.3)
Death at month 12, n (%)	5 (5.7)	1 (1.1)
Adverse events (at least one), n (%)	87 (100)	87 (100)
Serious adverse events (at least one), n(%)	69 (79.3)	68 (78.2)
Wound-healing delay, n (%)	2 (2.3)	1 (1.1)
Delayed graft function, n (%)	29[m] (34.9)	28[n] (34.6)
Lymphocele, n (%)	13 (14.9)	18 (20.7)
All infections, n (%)	46 (52.9)	42 (48.3)
Urinary tract infection, n (%)	27 (31%)	20 (23%)
BK virus DNAemia over the 12 months, n (%)	10[p] (15.4)	10[g] (14.3)
Post-transplant diabetes, n (%)	6 (6.9)	7 (8)
Anemia, n (%)	39 (44.8)	46 (52.9)
Neutropenia, n (%)	4 (4.6)	7 (8)
Thrombocytopenia, n(%)	6 (6.9)	1 (1.1)
12-months Triglyceride level (mmol/l), mean (SD)*	2.2[q] (1.1)	1.9[r] (1.2)
12-months HDL cholesterol level (mmol/l), mean (SD)	1.3[s] (0.4)	1.3[t] (0.4)
12-months LDL cholesterol level (mmol/l), mean (SD)	3.2[u] (1.1)	3.2[t] (1.1)

* p=0.03

[a] n=3, [b] n=2, [c] n=6, [d] n=9, [e] n=75, [f] n=74, [g] n=70, [h] n=71, [i] n=64, [j] n=66, [k] n=59, [l] n=60, [m] n=83, [n] n=81, [p] n=65, [q] n=54, [r] n=61, [s] n=50, [t] n=57, [u] n=51

Discussion

In this multicenter, randomized trial performed in R+ KTR not receiving CMV universal prophylaxis, CMV DNAemia occurred less frequently in patients of the EVR arm than in those of the MPA arm. Moreover, on-treatment analyses revealed that patients with an ongoing EVR treatment had an insignificant risk of CMV DNAemia requiring antiviral drug.

Recently, the large TRANSFORM and the ATHENA studies observed a lower incidence of CMV events in patients receiving EVR in association with low dose of CNI {Sommerer:2019ev} {Berger:2019cn}. The results of three randomized studies with CMV infection as primary endpoint were in accordance with these finding {Ferreira:2019hq, deSandesFreitas:2018fd, TedescoSilva:2015ev}. However, all these three studies were monocentric and included D+R- patients. Two of them used pp65 CMV antigenemia for the diagnosis of CMV infection instead of CMV QNAT. In spite of the already substantial literature on this subject, the EVERCMV study was original because it was based on a robust methodology: it was multicentric, followed a frequent monitoring of CMV QNAT calibrated with the WHO international standard for CMV, and included a homogenous population of R+ KTR. Based on these strengths, this study shows that *de novo* use of EVR is a strategy able to decrease CMV DNAemia in R+ KTR (6 months incidence, 39.2% with EVR vs. 77.8% with MPA). Moreover, the severity of CMV infections was also less important in patients of the EVR arm, with only 6.7% of treatment failure at 8 weeks in patients treated for CMV infection.

The discontinuation of EVR in some patients allowed us to observe that the incidence of CMV DNAemia was even much lower in patients with ongoing EVR (incidence, 21.4%) than in those who had stopped it or those of the MPA group. Importantly, maximal viral load of patients with ongoing EVR were very low and never above 5846 UI/ml. These low viral loads were

considered insignificant by the investigators who initiated treatment in only 7.4% of these patients. The interest of the preemptive strategy is to frequently screen for the appearance of CMV DNAemia in order to introduce antiviral treatment and prevent CMV disease. Since viremia observed under EVR were considered negligible by the vast majority of investigators and since only 4.1% CMV disease occurred in these patients, it can be concluded in hindsight that follow-up with CMV QNAT was unnecessary and could have been avoided. Until now, universal prophylaxis and preemptive therapy were the main approaches for prevention of CMV disease. Our findings support the idea to position EVR as new preventive strategy in R+ KTR as long as EVR can be continued.

There could be multiple anti-CMV effects of mTOR inhibitors. For instance, mTOR was shown to be essential for virus replication in myeloid cells {Poglitsch:2012bl, Nashan:2012ca}. However, mTOR inhibitors were also shown to increase both the quantity and the quality of virus-specific memory CD8 T cells in a mouse model of acute lymphocytic choriomeningitis virus infection {Araki:2009iq}, and the number of CMV-specific T-cell in R+ KTR {Gerna:2011gs}{Nashan:2012ca}{Havenith:2013is}{Bak:2018gp}. Our data indicate that the quantity of gamma-interferon produced by CMV-specific T-cells was increased rapidly after the initiation of EVR. Therefore, the improved functionality of CMV-specific T cells by EVR during the first two weeks post-transplantation could explain in part the preventive effect of EVR on CMV disease in R+ KTR.

The positioning of EVR in the preventive arsenal of CMV disease in R+ KTRs remains to be defined. Recently, it has been shown that recipients who had a protective anti-CMV cellular immunity at day 15 post-transplant were spared from post-transplant CMV infection {Jarque:2020fc}. For others patient, EVR could be proposed to avoid the use of valganciclovir,

which induces neutropenia in one quarter of patients in combination with MPA {Humar:2010fw} and exposes patients to rejection if MPA is discontinued {Zafrani:2009jc}. This strategy could be very pertinent and cost-effective in patients who do not develop EVR-associated side effects. In the 30% of R+ KTR patients who are forced to stop it due to side effects {SilvaJr:2010jx, Pascual:2018kq}, an universal prophylaxis or preemptive strategy could still be the best option.

De novo DSA, biopsy-proven acute rejection incidence, eGFR, graft and patients survival, and adverse events were similar between the two groups, in accordance with previous studies {SilvaJr:2010jx, Sommerer:2019ev}. Of note, others infection rates, and in particular BK virus DNAemia incidence were also similar between the 2 groups, contrasting with previous trials where EVR was shown to decrease BK virus nephropathy {Cibrik:2013gr, TedescoSilva:2019bs}.

Our study has some limitations. First, we were unable to include the initially planned number of subjects, but this did not prevent us from highlighting the protective effect of EVR with respect to CMV. Second, the protective effect of EVR has been demonstrated in combination with ciclosporin, whereas tacrolimus is considered todays as the best calcineurin inhibitor associated with lower rejection rate {Pascual:2018kq}. We chose cyclosporin because the trial was built before the TRANSFORM and ATHENA studies {Pascual:2018kq, Sommerer:2019ev}. Its design was based on the A2309 study {SilvaJr:2010jx}. Nevertheless, the comparison between the EVERCMV study and the post-hoc study of Tedesco-Silva et al. shows that the incidence of CMV DNAemia is identical between patients on cyclosporin and those on tacrolimus, whether in association with EVR (39.2% vs. 43.3%), or MPA (77.8% vs.

77.9%) {Basso:2018kb}. It is therefore reasonable to postulate that the anti-CMV preventive effect of EVR in treated patients is independent of the type of calcineurin inhibition.

In conclusion, EVR in association with calcineurin inhibitor not only reduces the incidence of CMV DNAemia but allows preventing the appearance of CMV DNAemia requiring antiviral drug treatment as long as it is tolerated and continued. This finding suggests that EVR could be considered as a new prevention strategy in R+ KTR.
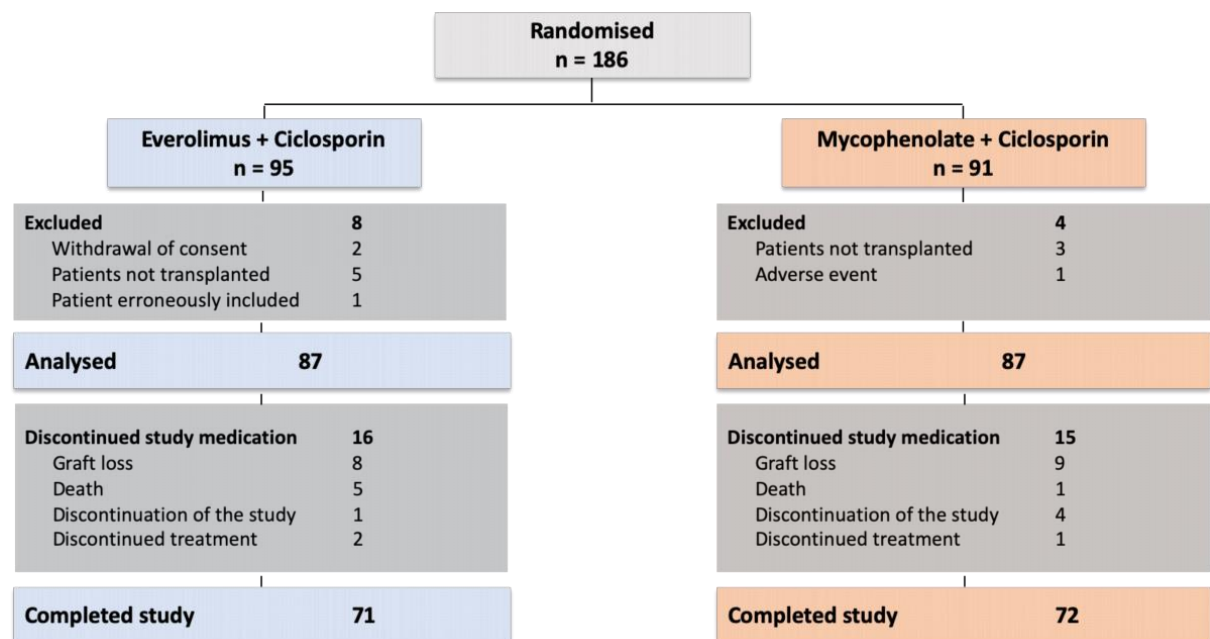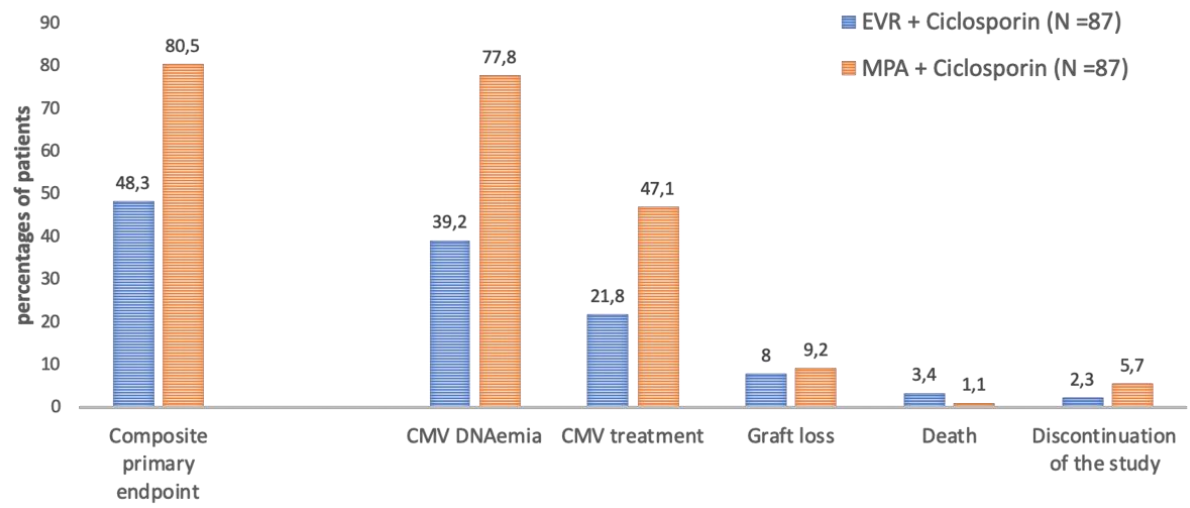
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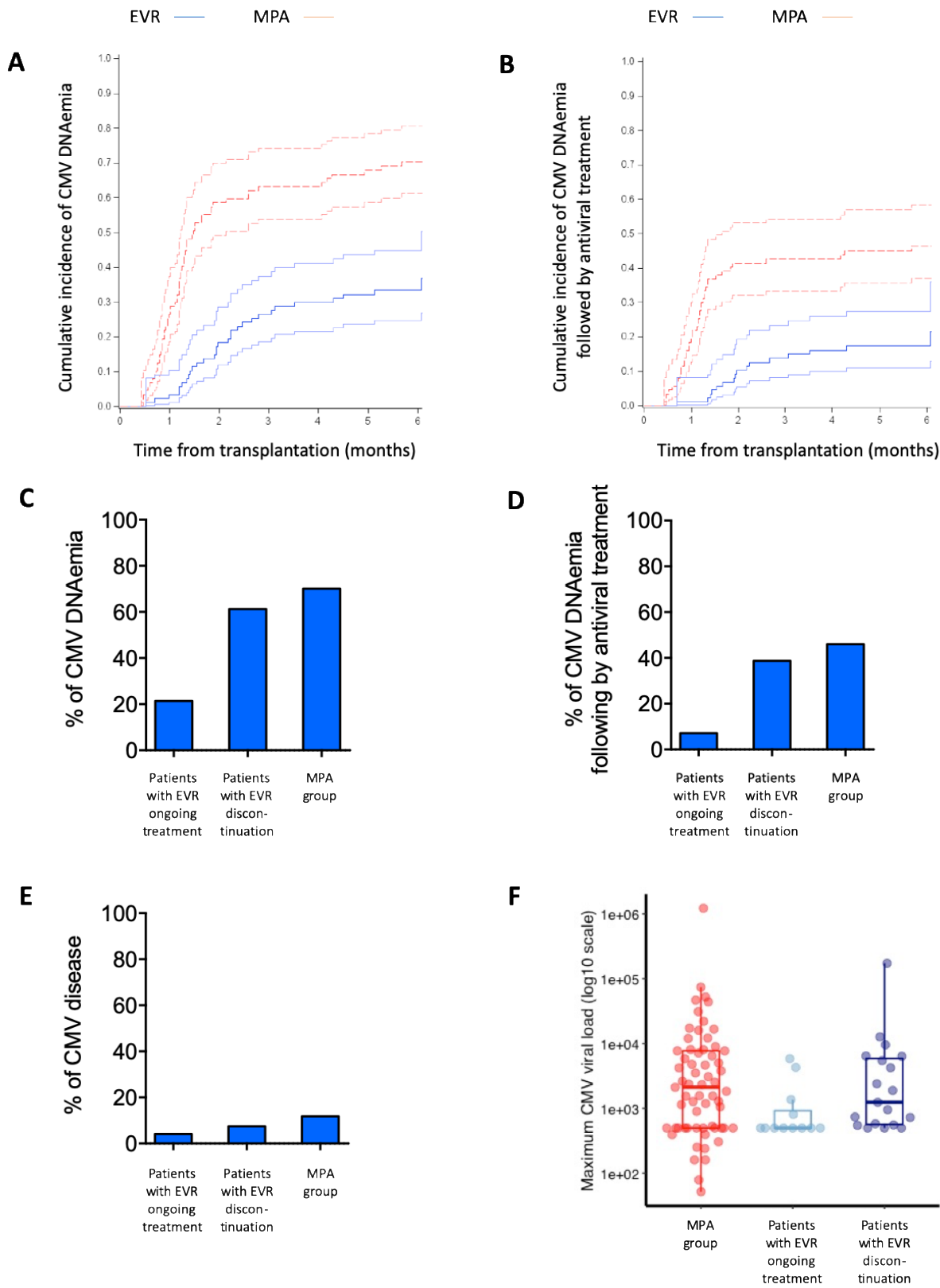
Figure 1

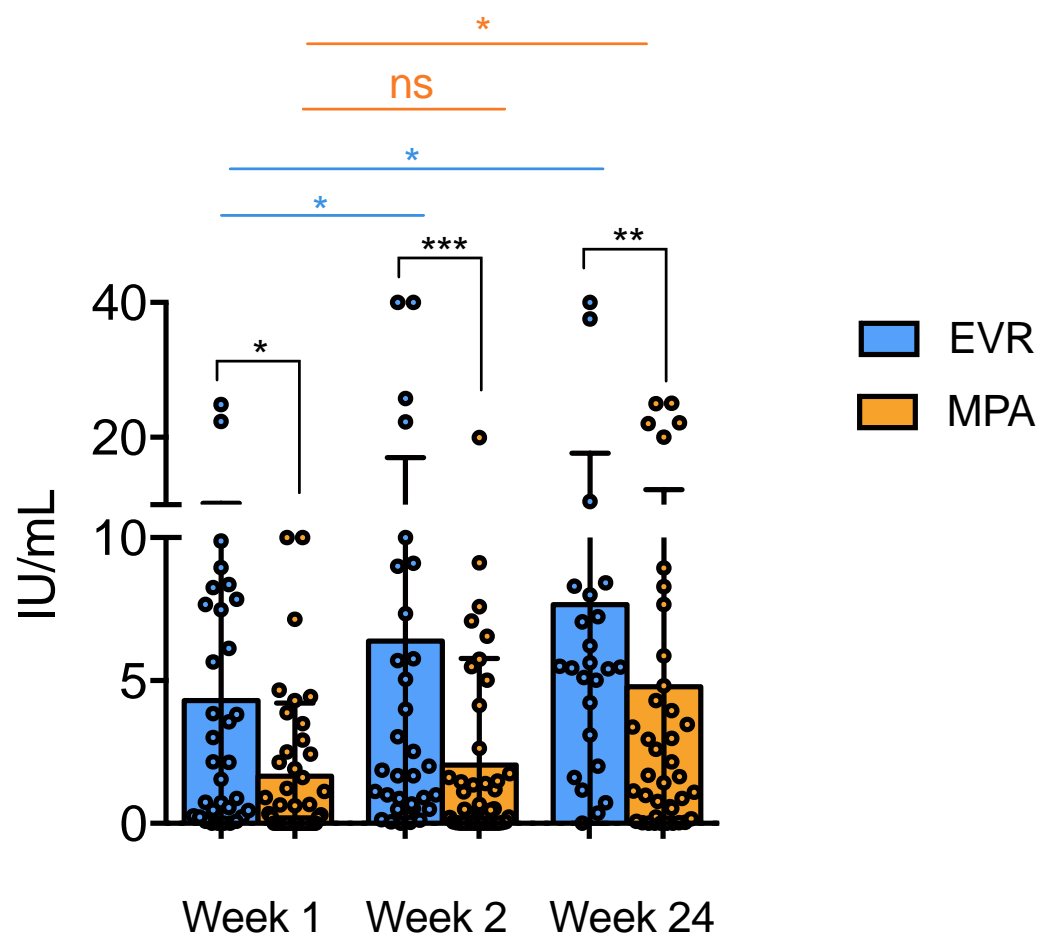**Figure 2**

Figure 3

Figure 4

Figure Legend

Figure 1. Flow chart of the study

The 186 CMV seropositive kidney transplant recipients fulfilling eligibility criteria were included and randomized to everolimus (EVR, n=95) or mycophenolic acid (MPA, n=91), in association with anti–IL-2 receptor antibody, cyclosporine and prednisone. Only 174 were analyzed (EVR group, n=87, and MPA group, n=87) and followed for one year.

Figure 2. Primary endpoint: intention-to-treat analyses

Proportions of events for the primary endpoint, which was defined as the composite of CMV DNAemia, CMV treatment, graft loss, death, and discontinuation of the study at 6 months post-transplantation, in patients of the everolimus group (n=87) and mycophenolate group (n=87). CMV DNAemia was defined as a single positive whole blood CMV QNAT.

Figure 3. CMV infection and disease incidence

One-year cumulative incidence curves of CMV DNAemia (A) and CMV DNAemia requiring antiviral drug treatment (B), among everolimus-treated patients (blue) and mycophenolate-treated patients (red).

One-year CMV DNAemia incidence among patients with ongoing everolimus treatment, patients with everolimus discontinuation and the mycophenolate group (C).

One-year incidence of CMV DNAemia requiring antiviral treatment among patients with ongoing everolimus treatment, patients with everolimus discontinuation and the mycophenolate group. The initiation of antiviral treatment was based on local standard practice (D).

One-year CMV disease incidence among patients with ongoing everolimus treatment, patients with everolimus discontinuation and the mycophenolate group. The initiation of antiviral treatment was based on local standard practice (E).

Maximal CMV QNAT among patients with ongoing everolimus treatment, patients with everolimus discontinuation and mycophenolate group. Median (range) are 499 IU/mL (499-5846) in patients with ongoing EVR treatment, 1244 IU/mL (499-173300) in patients with EVR discontinuation, and 2117 IU/mL (52-1 219 520) in MPA group (F).

Figure 4. CMV-specific cellular immunity

CMV-quantiferon was performed at day 7, day 14 and month 6 after transplantation and was compared between everolimus-treated patients and mycophenolate-treated patients Three conditions were used for each plasma : mitogen stimulation, CMV stimulation and medium alone (Nil). Nil results was subtracted to Mitogen and to CMV conditions. Then if CMV condition was >0.2IU/mL or <0.2IU/mL with Mitogen>0.5IU/mL, then the results obtained was given as a quantitative result. If CMV condition was <0.2IU/mL and mitogen <0.5IU/mL, quantiferon was considered as negative . ns, not significant, $0.05 > p > 0.01$*; **$0.01 > p > 0.001$ as determined by Mann-Withney U test.

DRAFT 4. Immunological exhaustion: How to make a disparate concept operational?

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Abstract
In this essay, we show that three distinct approaches to immunological exhaustion co-exist and that they only partially overlap, generating potential misunderstandings. Exploring cases ranging from viral infections to cancer, we propose that it is crucial, for experimental and therapeutic purposes, to clarify these approaches and their interconnections so as to make the concept of exhaustion genuinely operational.
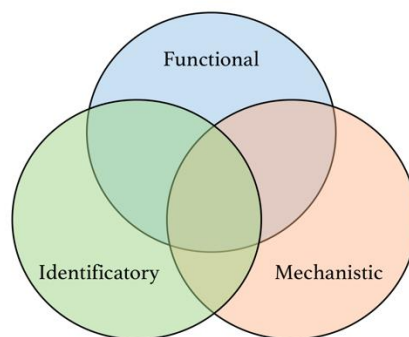
Fig. 1. Three different approaches to immunological exhaustion. These approaches use different criteria and do not always overlap.

1. Three approaches to exhaustion

Despite previous mentions to "exhaustion" in the immunological literature [1,2], the founding period for modern uses of the notion was the 1990s, during which exhaustion was defined both mechanistically and functionally focusing on specific CD8+ T cells during murine LCMV. "Exhausted" T cells are cells that, exposed chronically to high quantities of antigen, are activated and proliferate, before becoming dysfunctional, i.e., unable to eliminate the virus. For Zinkernagel and his team [3], exhaustion is defined by the non-elimination of the virus, due to the peripheral deletion of all the virus-specific cytotoxic T cells, a concept validated a few years later [4]. For Ahmed and his team [5], exhausted cells are dysfunctional because their capacity to trigger an effector response against the virus is reduced, but these cells are maintained in the body. Overall, exhaustion has classically been defined both by dysfunction (these T cells fail to do what effector T cells are expected to do) and by a double mechanism (high viral load and chronicity). Following their work, exhaustion has most of the time been applied to CD8 T cells, although it is also sometimes attributed to other immune cells (especially CD4 T cells) (e.g., [6]). Of note, both Zinkernagel's and Ahmed's groups worked on chronic lymphocytic choriomeningitis virus infection in mice (LCMV), which rapidly became, and still is today, the standard model for understanding exhaustion.

In subsequent research until present-day, three approaches to immunological exhaustion have co-existed, with often unclear connections (**Fig. 1**). The first approach primarily defines as exhausted the cells that are produced by a given *mechanism* (typically, but not necessarily, chronic exposure to an

antigen). The second approach primarily defines as exhausted the cells that present the same cellular *dysfunction* (typically, the absence of an expected effector response). Finally, the third approach primarily defines as exhausted the cells that present the same *molecular markers* (typically, PD-1). One difficulty is that authors do not always say which approach they have in mind when they qualify cells as "exhausted". A second, even more serious, difficulty is that authors often do as if these three approaches necessarily aligned (i.e., as if the three properties always occurred together), when in fact they don't. More recently, a subset of stem-like CD8+ T cells has been identified among exhausted T cells but the non-overlap also apply for it [7–10].

Especially, antigen chronicity has been quite considered as the consensual mechanism leading to exhaustion whereas even in the LCMV model, this statement could be questioned. Bellow, we give several examples of non-overlap, and we insist on the importance of both distinguishing and combining these three approaches to build a precise and operational account of exhaustion.

In our view, the lack of articulation between the three approaches distinguished above is the prime explanation for the current ambiguities and disagreements around the notion of exhaustion, as exemplified recently in the various and often competing positions expressed by 19 experts [11]. **Fig. 2** sums up the main controversies about exhaustion.
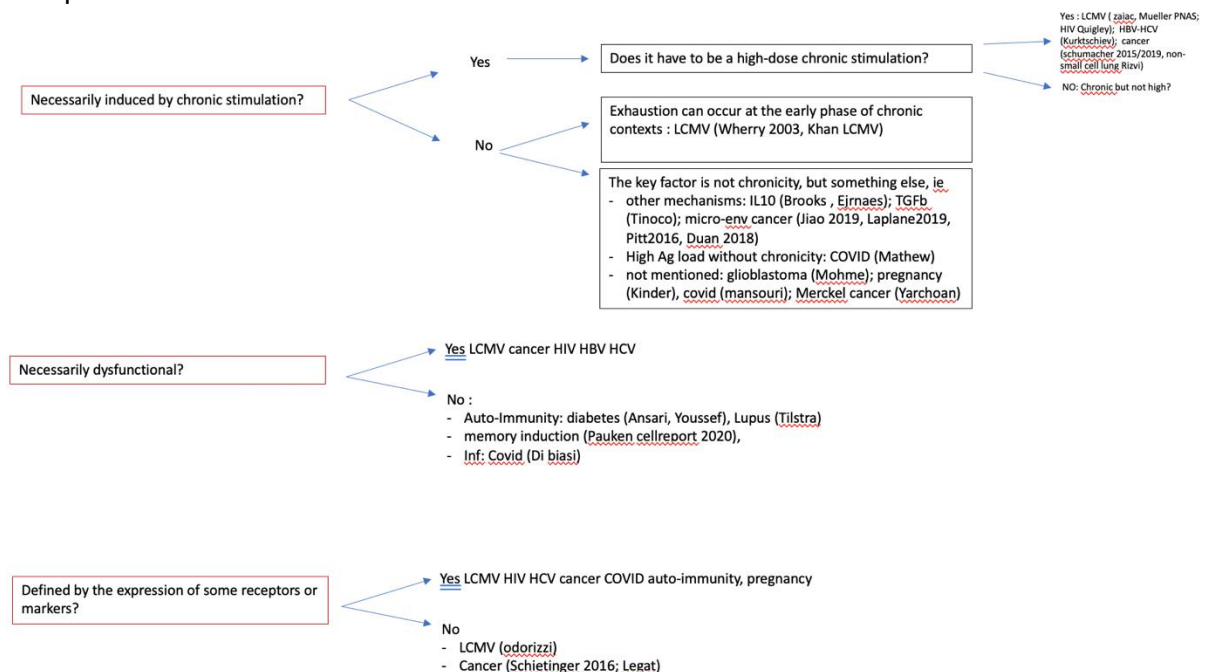

Fig. 2: Key controversies about immunological "exhaustion"

Because we propose that it is essential for immunologists to distinguish the three approaches and make explicit in each experimental or therapeutic context which approach(es) they have in mind, we try in what follows to present as precisely as possible these three approaches.

1.1. The functional approach: exhaustion is a cellular dysfunction

Originally, exhausted T cells were understood as dysfunctional. What is meant by "dysfunction" here is that some T cells, after activation and proliferation, do not fulfil the functions they are expected to perform as effector T cells – typically, they fail to eliminate infected cells and control the virus. As originally described, Ag-specific T cells become "dysfunctional" during the chronic phase of high viral

load infections, with progressive loss of IL-2, then TNFα, and finally IFNγ. Authors have called these cells "dysfunctional" because they compare their functions in the context of a high-dose persistent chronic viral infection to the functions of the same cells in the context of acute viral infection when the virus is cleared [1].

It is essential for the rest of this paper to understand that, in the original sense of "exhaustion" and especially for Zinkernagel's team, the dysfunction that is characteristic of exhaustion can be *defined* independently of any mechanism or marker. Exhaustion is understood as the non-elimination of the virus due to the peripheral deletion of virus-specific cells. This constitutes a genuine functional characterization of exhaustion. Within this approach, saying that exhaustion results from a mechanism of exposure to high doses of a chronic antigen is therefore not circular: this mechanism is offered as the best possible explanation for an independently defined phenomenon.

The dysfunction associated with exhaustion has often been conceived in contrast to another dysfunctional state, namely "anergy". The consensus is that anergy results from an absence of signal 2, and therefore a failure to become activated, while exhaustion occurs after T cells have been fully activated and have proliferated [12–14].

It is important to characterize as precisely as possible the nature of the dysfunction thought to be characteristic of exhaustion. For example, although this dysfunction has sometimes been conceived as involving an incapacity for maintenance and expansion [15–17], it has in fact been observed that some T cell populations with an exhausted phenotype in chronic infections can maintain and expand [13], iethe stem-like CD8+ T cell exhausted T cell subset (for review, see [18]). In this line, we now know that the exhaustion of such stem-like cells and at least in some situations, is reversible [12], contrasting with the long-held view that exhaustion would necessarily be irreversible [3,19–21]. Especially, the reversibility seems to concern the TCF1+ cells among exhausted cells. These PD-1+CD8+ T cells resemble stem cells during chronic LCMV infection, undergoing self-renewal and also differentiating into the terminally exhausted CD8+ T cells [9]. However, IRF4 inhibition seems also able to induce TCF1 and leads to exhaustion reversibility [22].

"Exhausted" cells transferred into a naïve host can re-expand and protect it against the same pathogenic challenge [23], suggesting that such cells are not irreversibly "dysfunctional", and this restoration is obtained is now known obtained through the stem-like cells [9]. However, the dysfunction of stem-like cells concerns maintenance, expansion and differentiation but not cytokine production (IFNγ) and cytotoxicity (granzyme B) since the terminally exhausted cells coming from the stem-like ones have the more cytotoxic potential [9].

Is exhaustion always dysfunctional? There are important disagreements about this question, some authors considering that exhaustion is by definition dysfunctional, while for others exhaustion could be functional in some contexts [11,23,24]. One major limitation is that, in general, exhausted T cells are said to be "dysfunctional" with a unique and preexistent idea of "function" in mind – namely the function to eliminate the virus. This expected and non-realized function remains in almost all cases the unique focus of the observer, which means that other active functions, such as immunoregulation or tissue-repair for example, are generally not explored. This makes it impossible to determine if, by coming "exhausted", cells lose some functions while simultaneously acquiring other functions. Major examples of contexts where so-called "exhausted" T cells could in fact play essential functional roles include the limitation of immunopathology in infection [25] and the regulation of autoimmunity [26].

1.2. The mechanistic approach: exhaustion as resulting from specific mechanisms

A second major approach is to say that exhausted cells are cells that are produced by a particular mechanism, typically antigenic chronicity and/or high antigen load. In the original LCMV model [3,5], both chronicity and antigen load were considered essential mechanisms leading to exhaustion.

In subsequent work, antigenic chronicity remained an often-mentioned typical mechanism leading to exhaustion [21,27] . Naturally, an important challenge when saying that exhaustion results from chronic exposure to an antigen is to determine what is meant by "chronic", i.e., what the exact time course of exhaustion appearance is. Despite the general consensus that exhaustion is related to antigen chronicity, recent results have shown that this connection was not always there. Although exhaustion has been classically observed during the chronic phase of uncleared viruses, recently it was shown that, as early as 9 days post-infection of C13 LCMV, CD8 T cells lose their abilities to make TNFα and IL-2 [28,29], which seems a very short time to talk about "chronicity". One hypothesis here could be that Tox expression represents an early fate decision, but not demonstrated yet? Still recently, high dose of C-13 (10^6) in contrast to low-dose of the same virus (10^2), leads to exhausted cells by day 8 [9], suggesting that the load rather than the chronicity of antigen-exposure matters for T cell exhaustion.

A second mechanism said to trigger exhaustion is high antigen load. As already said, in the historical LCMV model, the claim that T cells were exhausted in C-13 by contrast to the Armstrong strain was based on the fact that the viral load was both higher and chronic [5,21]. The insistence on the role of a high antigen load also has roots in the concept of "immune paralysis". This concept, widely used in the 1960-70s [2,30,31], proposed that the immune system could become inoperant when confronted to high doses of antigen [21,28]. Later, some papers insisted on the crucial importance of high antigen load in the characterization of exhaustion, with or without an accompanying mention of chronicity [21,29,32]. A key challenge for this approach is to determine what quantity of antigen constitutes a "high dose", which is often very difficult to do [21].

Importantly, other mechanisms of exhaustion, beyond chronicity and antigen load have been suggested including contextual mechanisms. One such additional mechanism is the cytokine environment, with a typical focus on IL-10 and TGFβ (which tend to favor exhaustion) [29,33,34] as well as IL-21 (which, in contrast, tends to inhibit exhaustion) [35]. Incidentally, the impact of the cytokine environment on the functionally defined exhausted phenotype of CD8 T cells demonstrates that these cells are not intrinsically "exhausted"; instead, exhaustion in the functional sense appears to be context-dependent. Exhaustion is also influenced by mechanisms at the cellular level, including both regulatory T cells [36], [37] and myeloid-derived suppressor cells [38].

1.3. The identificatory approach: exhaustion as the cellular state associated with the expression of particular molecular markers

According to the third approach, exhaustion is the cell state associated with the expression of particular molecular markers – classically inhibitory receptors. There are many of them, so only the most discussed in the scientific literature are mentioned here.

PD1 has been the most studied marker of exhaustion. It is considered to characterize exhausted T cells in both chronic infection and cancer, and associated with dysfunction in both cases [39,40]. In addition to PD-1, there are other receptors often considered as markers of exhaustion, often but not always in association with PD-1 (LAG3, TIM3, TIGIT, CTLA4, etc.).

Some transcription factors have been interpreted as favoring exhaustion, for example Blimp-1, Eomes, Tox, and IRF4 (reviewed in [41]). During chronic infection, Eomes has been correlated with

the "more terminal T_{ex} subset" [42]. Tox has been considered as a key transcription factor of the T cell exhaustion program, since it was highly expressed in T cells during C-13 LCMV infection, whereas it was only transiently expressed at low levels during acute infection with Armstrong [28]. Moreover, T cell exhaustion in cancer and chronic infection mainly relies on the presence of Tox, itself driven by chronic TCR stimulation and NFAT activation, and associated with the expression of other transcription factors that are required for Tex cells (TCF1, Eomes), as well as with inhibitory receptors and decreased function [28]. IRF4 contributes to induce inhibitory receptors and leads to decreased functionality of CD8+ T cells during the chronic phase of LCMV infection and during cancer [22,43,44].

Finally, exhausted cells display metabolic changes such as inhibition of aerobic glycolysis due to glucose transport limitation and consumption by cancer cells, mitochondrial dysfunction and oxygen deprivation [45], which in turn decreases cytokine production [46].

2. The incomplete overlap between the three approaches to exhaustion

A common attitude is to assume that the three approaches to exhaustion generally align: dysfunction would be produced by well-identified mechanisms such as chronicity and/or high antigen load, and it would be associated with the expression of well-identified markers such as PD-1. In reality, though, these three approaches often do not overlap.

Many data suggest that exhaustion in the functional sense does not always result from the mechanism of antigen chronicity. Exhausted T cells have been described for chronic infection with LCMV, HIV, HBV and HCV but not for CMV, for example. Even if kinetic of viral load is hypothesized as mechanistically involved to explain either exhaustion during LCMV and inflation during CMV, a better understanding of the mechanism involved is needed (CMV is thought to give series of low and short multiple replication periods contrasting with high chronic level of replication during LCMV but studies on CMV suggest that the virus could still replicate actively in the tissues whereas is negative in peripheral blood [47]). Moreover, the analysis of the profile evolution of CD8+ T cells in several infections shows that exhaustion in the sense of a dysfunctional phenotype occurs during the acute phase of C-13 LCMV infection, but not in other infections such as influenza, VSV, or *Listeria monocytogenes* [28]. In a number of cases, antigen chronicity has been shown to be only partially related to exhaustion in a functional sense: depending on the epitope, load, and duration of infection, antigen-specific CD8 T cells responding to chronic antigen exposure may be fully functional, partially exhausted, fully exhausted, or physically deleted [21]. In other instances of exhaustion, we simply don't know if antigenic chronicity plays a role or not. For example, in the analysis of the phenotypes of infiltrating T cells and peripheral blood of patients with non-small cell lung cancer, hepatocellular carcinoma or glioblastoma [28,48], or during pregnancy [49], we don't have a kinetic approach that would allow us to say if the chronic exposure to tumor- or fetal-antigens played a role in the induction of exhaustion.

Another important and related challenge is that exhaustion in the functional sense does not always result from the mechanism of exposure to high doses of antigen. It was recently shown that, in different infections, the adoption of an "exhausted" dysfunctional phenotype by antigen-specific CD8+ T cells occurred before the viral outcomes diverged, suggesting that viral load was not a primary driver of differential expression [28].

Regarding the markers used to define exhaustion, absence of overlap also exists. First, although PD-1 is the most often mentioned inhibitory receptor associated with exhausted T cells, it is certainly not a specific marker of exhaustion, as it is expressed after acute TCR activation [50]. The expression of PD-1 by non-exhausted cells is a widely recognized phenomenon [51]. Minimally, this forces us to recognize that PD-1 might be a necessary, but not a sufficient, marker of exhaustion, and

that exhaustion corresponds more to a cluster of several markers rather than just one marker. Moreover, PD-1 is not always required for the induction of exhaustion, and some features of exhaustion can even be more severe when PD-1 is absent [51]. Finally, the role of PD-1 has been highlighted in many contexts other than exhaustion, including autoimmunity [52], central and peripheric tolerance [53,54], acute infection for memory response [55], and balance between efficient anti-infectious defense and immunopathology [56]. In those contexts, PD-1 was not related to cellular dysfunction, and was even associated with an increased memory response function [55,57]. Thus, the function of PD1 is interpreted differently depending on where and when it has been studied – sometimes as a sign of "exhaustion", sometimes not. It seems that the most appropriate description would be to say that PD-1 is expressed in contexts of *inhibition*, rather than contexts of *exhaustion* per se, in so far as "exhaustion" is generally used when PD-1 is expressed in a context that is detrimental to the organism, but not when the context is beneficial (e.g., in peripheral tolerance during auto-immunity [58], or during T cell memory formation [55]. Conversely, anti-PD1 therapy has been associated with the development of auto-immune diseases such as type-1 like diabetes [59].

Transcription factors also have ambivalent roles depending on the context. For example, Eomes has been associated with exhaustion during the chronic phase of infection , but it is upregulated during acute infection, favoring effector molecule production (IFNγ), IL15Rβ, and memory development [42,60]. Tox expression, highlighted as a key component of the exhaustion program, can in fact be already observed at day 4 of infection before antigen loads differ [28], which suggests that neither the chronicity nor the viral load are primary drivers of its expression. Moreover, the deletion of Tox is correlated with downregulation of inhibitory receptor gene expression and this phenotype rescues the polyfunctional profile transiently but not in the long term, weakening the idea that this marker necessarily overlaps with dysfunction. Since the mechanism mentioned by the authors was chronic TCR stimulation, they hypothesized that the TOX program serves to prevent overstimulation of T cells and immunopathology [39,61], which constitutes an alternative function rather than a dysfunction properly speaking. Consequently, the role of the differential expression of Tox in C13, Armstrong and other microbial infections remains poorly understood.

IRF4 has been described both in LCMV infection and in allotransplantation, with a potential dual role. It shows a pro-exhaustion role during LCMV and cancer. In contrast, the deletion of IRF4 in CD4 T cells resulted in dysfunction and graft tolerance, which means that, when expressed, IRF4 represses PD1 and Helios [62], and favors reactivity to the allograft.

Finally, the metabolic changes observed in exhausted T cells can also occur in the tumor microenvironment through mechanisms distinct from antigen chronicity and high antigen load [63].

The upshot is that, despite the frequent tendency to do as if the three approaches to immunological exhaustion are aligned, this is often not the case. Some situations can be described as exemplifying "exhaustion" if seen from the functional viewpoint, but are not associated with the classic molecular markers of exhaustion and/or the classic mechanisms of exhaustion. This absence of convergence between the three approaches can be extremely problematic, as we illustrate now with the examples of cancer and Covid-19.

3. Two illustrations of issues raised by incomplete overlap: Cancer and Covid-19

The fact that the three approaches to exhaustion do not always conceptually overlap can generate significant issues at the experimental and clinical levels. The application of the notion of "exhausted T cells" to cancer is telling.

The aim of cancer therapies based on immune checkpoint inhibitors is to target dysfunctional T cells in cancer by reversing their state of tolerance to the tumor [64,65]. In recent years, many have

connected the literature on immunotherapies with the preexisting literature on immunological exhaustion, and claimed that targeting dysfunctional T cells in cancer amounted to targeting exhausted T cells [66–69]. This claim, however, is problematic for at least two fundamental reasons.

First, for the clinician, targeting "exhausted T cells" will have a very different meaning depending on whether what is targeted is the dysfunction itself, the underlying mechanisms, or the markers of exhaustion (**Fig. 3**). Doing as if the three approaches always overlapped in cancer would be inappropriate and misleading, as *1)* there are cases of cancer where some markers of exhaustion are dissociated from the mechanisms of high antigen load and chronicity [70], *2)* dysfunction sometimes occurs without the expression of traditional markers of exhaustion [71], and conversely *3)* traditional markers of exhaustion are sometimes expressed in functional cells [72]. This confirms the importance of systematically specifying which meaning of "exhaustion" one has in mind when suggesting to target exhaustion in a cancer setting.
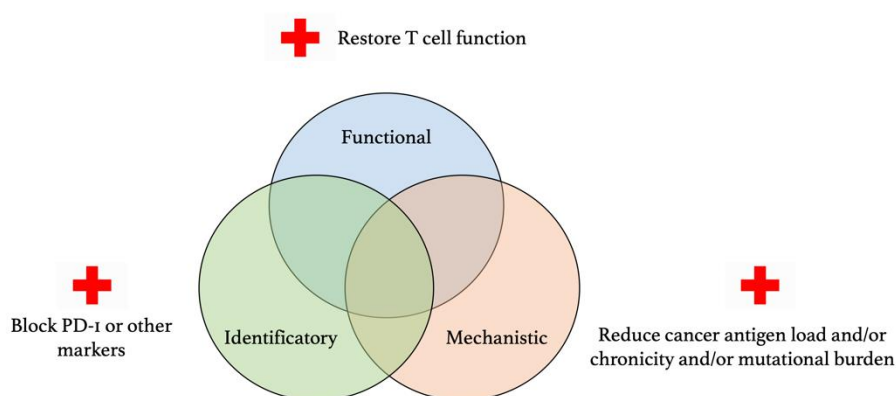

Fig. 3. Targeting "exhaustion" in cancer therapies means different things depending on the approach to immunological exhaustion one adopts.

Crucially, even when focusing on one specific and explicitly described approach to exhaustion, many important difficulties remain. This can be illustrated with the application to cancer of the mechanistic approach of exhaustion. The main suggestion in papers linking cancer and exhaustion has been that the mechanisms characteristic of exhaustion in the viral context would also occur in cancer: the chronic exposure of T cells to a high load of tumor antigens would lead to their exhaustion, and therefore to the incapacity of these T cells to control tumor growth. This idea led to the hypothesis that poor immune responses to cancer are related to chronic exposure to high levels of tumor "neoantigens" [73,74] (typically seen as a consequence of a high rate of genetic mutations [75,76]). In clinical practice, the expectation has been that a high mutation load would lead to exhaustion, and therefore to good responses to anti-PD1 therapies. This is indeed what is seen in some cancer types [77]. For example, neoantigen burden in non-small cell lung cancer is directly correlated to the clinical response to anti-PD-1 therapy [78]. Yet, in a number of other cancer types, the correlation does not hold. Some cancers, such as the renal cell carcinoma, have a response to anti-PD-1 therapy that is better than would be predicted by the tumor mutational burden. Others, such as colorectal cancer with mismatch repair proficiency have a response that is worse than would be predicted by the tumor mutational burden [77]. Overall, assuming an overlap between dysfunction and mechanisms of chronicity and high antigen load to predict T cell exhaustion and thus anti-PD1 response would lead to unsuitable therapeutic strategies.

Second, even in the limited number of cases in which there is a satisfactory overlap between the three approaches to immunological exhaustion (dysfunction, mechanisms, markers), focusing on exhaustion remains problematic because exhaustion constitutes at best a small subset of the many

forms of immunological tolerance to the tumor. There are, in fact, many parameters that help explain the elimination or non-elimination of the tumor by T cells, and each of these parameters can be linked to biomarkers – as suggested by the concept of the "cancer immunogram" [79]. Furthermore, even the focus on CD8 and CD4 T cells is increasingly recognized as too narrow: the nature of the immune response to tumors also depends on innate immune cells such as macrophages, and on the tumor micro- and macro-environment [80–83], which all impact T cells' function and also play T cell-independent roles. Thus, from a clinical point of view, not only one must keep in mind that the concept of exhaustion takes different and often non-overlapping meanings, but it is also essential to consider that immunological tolerance, be it mediated by "exhaustion" or not, is always dependent on "contextual" factors, some of which can be mechanistically studied and therapeutically manipulated.

Recent work on Covid-19 offers another interesting example of the potential inconsistencies and misunderstandings that can be associated with the concept of exhaustion. CD8+ T cells present an exhausted phenotype in many Covid-19 patients, but the mechanism involved is more high antigen load than chronicity, since the markers and the dysfunction of CD8+ T cells were observed at day 7 of infection which, again, is a short period [84,85]. Even more importantly, it is now well-known that patients can die from an overstimulation of the immune system, especially via "cytokine storms" [86], also illustrated by the fact that 10% of severe COVID-19 was observed in patients with neutralizing auto-antibodies against I IFN which decrease initial I IFN, lead to viral spread and later to hyperinflammation [87], another mechanism in which exhaustion is not involved. Consequently, misunderstanding "the exhausted-profile" as an indiscriminate need for T cell reinvigoration could lead to clinical disasters for the patients. As with cancer, a productive application of the conceptual framework of exhaustion to Covid-19 will require a precise examination of the three approaches (that is, in terms of dysfunction, mechanisms, and markers) and a careful reflection on the full therapeutic consequences of the manipulation of "exhausted" T cells.
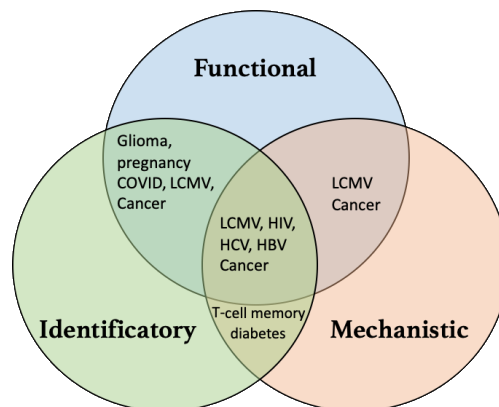

Fig.4. Same schema as Fig. 1, but with examples of non-overlap.

4. Conclusion: Exploring new avenues about exhaustion and immunoregulation

From the 1990s to the present day, many immunologists have suggested that exhaustion might in fact reflect an evolutionary conserved mechanism of immunoregulation [5,11], including as a means to limit immunopathology. Indeed, what immunologists have dubbed "exhaustion" to initially describe cells that seem to fail to realize their expected effector functions may well actually be one manifestation of a sometimes beneficial physiological process, preventing excessive immune responses and excessive damages.

One important way to approach this question is to adopt a multi-level perspective. A process that seems dysfunctional at a lower level can in fact be part of a functional regulatory process at a higher level. Typically, a state of exhaustion might be dysfunctional at the cell level while being functional at the tissue or organism level, for instance if exhaustion helps limit immunopathology. This approach is convergent with a role for exhaustion in the phenomenon of "disease tolerance". Disease tolerance, a term long used in plant ecology [88] and referring to a reduction of the negative impact of an infection on host fitness without directly affecting the pathogen burden, has been increasingly studied in recent immunology [89,90]. The phenomenon of disease tolerance reflects the fact that, in terms of fitness, it is sometimes better to mitigate the impact of a source of damage rather than eliminate it. Immunological exhaustion has indeed be characterized as one of the mechanisms by which disease tolerance is achieved [91]. Future research will help clarify the concept of exhaustion thanks to a better understanding of its connections with both "disease tolerance" (which has to do with the non-elimination of a source of damage) and "immunological tolerance" (which has to do with the downregulation of an effector immune response).

The perspective presented in this paper opens up original and promising avenues for future research, in at least four areas:

i) Regulation to self: future work needs to determine whether immune responses to "self" display some characteristics generally associated with exhaustion (in this case, people will presumably prefer to talk about "regulation"). Recent research has started to explore the role of exhaustion in the prevention of autoimmune diseases [26,92], but more work on this issue is needed, and the role of exhaustion must be investigated not only in autoimmune diseases but also in physiological autoimmunity (i.e., responses to "self", for example in tissue maintenance and tissue repair). We expect that the experimental inhibition of exhaustion will favor autoimmune diseases, allergy and inflammatory diseases, as well as dysregulation of physiological responses to self-constituents.

ii) Regulation to "resident nonself": typically, the microbiota. Studies on the microbiota do not even examine the phenomenon of immunological exhaustion. An intriguing hypothesis would be that the experimental inhibition of exhaustion in the gut or on the skin might lead to elimination of some microbes with which the host normally cohabits.

iii) Negative consequences of excessive limitation of exhaustion in contexts of infection: although limiting exhaustion in some contexts of viral infection is a legitimate objective, we expect that an excessive inhibition of exhaustion could lead to pathological consequences, from immunopathology to the development of autoimmune disorders. This idea of an equilibrium between beneficial and detrimental exhaustion has been present for a long time in the literature on exhaustion and has recently been specifically investigated, but what is needed now is an explicit description of the mechanisms that will insure an adequate equilibrium.

iv) Negative consequences of excessive limitation of exhaustion in cancer: there is no doubt that reversing T cell exhaustion in some cancers can be useful and even sometimes save lives. Yet, as we saw, it is not always clear in which sense these cells should be considered "exhausted", and in many cases restoring the functions of these cells does not really amount to suppressing their state of "exhaustion". Moreover, future research will certainly confirm that the "reinvigoration" of T cells in cancer may lead to detrimental consequences for the host in terms of immunopathology and autoimmune responses, which makes it all the more important to i) specifically understand the type of immunoregulation (not necessarily reducible to exhaustion) involved in immune responses to cancer, and ii) systematically understand mechanisms of T cell responses to cancer in the broader context of the many components of the immunological tumor microenvironment.

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Titre : Physiologie des lymphocytes T gamma-delta dans l'interaction du cytomégalovirus avec son hôte immunodéprimé

Résumé : Le cytomégalovirus est une cause infectieuse majeure de morbi-mortalité après une transplantation rénale. Une meilleure connaissance des acteurs du système immunitaire impliqués dans la réponse contre le CMV et de l'effet des médicaments immunosuppresseurs sur ces acteurs permettrait d'améliorer la prise en charge des patients. Nous avons précédemment démontré que les lymphocytes T $\gamma\delta$ (LT$\gamma\delta$) (et plus particulièrement les populations n'exprimant pas la chaine Vδ2 du TCR) avaient des caractéristiques de cellules adaptatives et étaient des cellules effectrices clés répondant au CMV et associées à la guérison. Dans un premier temps, nous avons analysé le répertoire et les fonctions des lymphocytes T $\gamma\delta$ Vδ2neg naïves pour mieux connaître leurs propriétés "innées" grâce à une analyse transcriptomique à l'échelle de la cellule (single cell RNASeq). Deuxièmement, un sous-groupe de LT$\gamma\delta$, les Vγ9negVδ2pos ayant également des caractéristiques adaptatives a été récemment décrit chez l'adulte. Nous avons montré que ces LT$\gamma\delta$ Vγ9negVδ2pos sont également des composants de la réponse immunitaire contre le CMV tout en présentant des caractéristiques distinctes de celles des LT$\gamma\delta$ Vδ2neg. Notamment, les LT$\gamma\delta$ Vγ9negVδ2pos étaient le seul sous-groupe de LT$\gamma\delta$ dont l'expansion était corrélée à la gravité de la maladie à CMV. Par conséquent, ce travail évalue un nouvel acteur dans la réponse immunitaire contre le CMV et ouvre des perspectives cliniques intéressantes pour l'utilisation des LT$\gamma\delta$ Vγ9negVδ2pos comme immuno-marqueur de la gravité de la maladie à CMV. Enfin, nous avons analysé l'effet des inhibiteurs mTOR (mTORi), traitement anti-rejet utilisé en transplantation rénale, sur les lymphocytes T spécifiques du CMV. En effet, les mTORi sont associés à une moindre incidence d'infection à CMV chez les receveurs séropositifs (R+) de greffes de rein (KTR) mais leur impact sur la réponse T n'est pas connu. Nous avons émis l'hypothèse que la réactivation du CMV chez les patients R+ pourrait être due à une dysfonction des lymphocytes T qui pourrait être améliorée par les mTORi. Nous avons d'abord montré que les lymphocytes T alpha-bêta et gamma-delta présentaient un phénotype plus dysfonctionnel (LAG3+, TIM3+, PD-1+, CD85j+) à la transplantation chez les 16 R+ KTR chez qui survenait une réactivation sévère du CMV par rapport aux 17 patients sans reactivation ou avec une infection spontanément résolutive. Les patients sous mTORi (n=27) avaient une proportion diminuée de lymphocytes T alpha-beta et gamma-delta PD-1+ et CD85j+ par rapport aux patients traités par acide mycophénolique (MPA) (n=44), ce qui était corrélé avec une fréquence et une gravité moindre des infections à CMV. Les patients sous mTORi présentaient également des proportions plus élevées de lymphocytes T cytotoxiques. In vitro, les mTORi augmentaient la prolifération, la survie et la production d'interferon-gamma contre le CMV par les lymphocytes T alpha-beta et gamma-delta. Les proportions de cellules PD-1+ et CD85j+ étaient également diminuées sous mTORi dans les deux sous-populations et leur profil majoritaire devenait "EOMES low/ Hobit high". Dans les lymphocytes T gamma-delta, l'effet des mTORi était lié à une augmentation de la signalisation TCR. Ces résultats révèlent (i) qu'une réplication sévère du CMV est associée à un profil dysfonctionnel des lymphocytes T et (ii) que les mTORi améliorent leur aptitude de façon associée au meilleur contrôle du CMV. Le phénotype de lymphocytes T dysfonctionnel pourrait représenter un nouvel immuno-marqueur chez les patients R+ pour prédire l'infection post-transplantation et aider à stratifier les patients qui devraient bénéficier du traitement mTORi.

Mots clés : Lymphocyte T gamma-delta, CMV, transplantation rénale, inhibiteurs de mTOR

Title : Insights into the physiology of $\gamma\delta$ T lymphocytes through CMV/ immunocompromised host interaction study

Abstract : Cytomegalovirus is a major cause of morbidity and mortality after kidney transplantation. A better understanding of the actors of the immune system involved in the response against CMV and of the effect of immunosuppressive drugs on these actors would improve patient management. We have previously demonstrated that $\gamma\delta$ T lymphocytes (LT) (and more specifically populations not expressing the Vδ2 chain of TCR) had adaptive characteristics and were key effector cells responding to CMV and associated with patient recovery. First, we analyzed the repertoire and the functions of naive Vδ2^{neg} LT$\gamma\delta$ to better understand their "innate" properties through transcriptomic analysis at the cell level (single cell RNASeq). Secondly, a subgroup of LT$\gamma\delta$ Vγ9^{neg}Vδ2^{pos} also having adaptive characteristics has recently been described in adults. We have shown that these Vγ9^{neg}Vδ2^{pos} LT$\gamma\delta$ are also components of the immune response against CMV while exhibiting distinct characteristics from those of Vδ2^{neg} LT$\gamma\delta$. Notably, the LT$\gamma\delta$ Vγ9^{neg}Vδ2^{pos} was the only subgroup of LT$\gamma\delta$ whose expansion was correlated with the severity of CMV disease. Therefore, this work evaluates a new actor in the immune response against CMV and opens interesting clinical perspectives for the use of LT$\gamma\delta$ Vγ9^{neg}Vδ2^{pos} as an immune marker of CMV disease severity. Finally, we analyzed the effect of mTOR inhibitors (mTORi), a treatment to avoid graft rejection used in kidney transplantation, on CMV-specific T cells. Indeed, mTORi are associated with a lower incidence of CMV infection in CMV positive (R+) kidney transplant recipients (KTR), but their impact on the T cell response is unknown. We hypothesized that the reactivation of CMV in R+ KTR may be due to T-cell dysfunction that could be improved by mTORi. We first showed that LT$\alpha\beta$ and LT$\gamma\delta$ had a more dysfunctional phenotype (LAG3+, TIM3+, PD-1+, CD85j+) before transplantation in the 16 R+ KTR patients who will undergo severe CMV reactivation after transplantation compared to the 17 patients without reactivation or with well-controlled CMV infection. Patients on mTORi (n=27) had a decreased proportion of PD-1+ and CD85j+ $\alpha\beta$ and $\gamma\delta$ LT compared to patients treated with mycophenolic acid (MPA) (n=44), which correlated with a lower frequency and severity of CMV infections. Patients on mTORi also had higher proportions of cytotoxic T cells. In vitro, mTORi increased the proliferation, survival and production of CMV interferon-gamma by $\alpha\beta$ and $\gamma\delta$ LT. The proportions of PD-1+ and CD85j+ cells were also decreased under mTORi in both subpopulations and their profile became mainly "EOMES low/ Hobit high". In LT$\gamma\delta$, the effect of mTORi was related to an increase in TCR signaling. These results reveal (i) that severe CMV replication is associated with a dysfunctional T cell profile and (ii) that the mTORi enhance their ability to control CMV replication. The dysfunctional T-cell phenotype may represent a novel immune marker in R+ patients to predict post-transplant CMV infection and helps stratify patients who should benefit from mTORi therapy.

Keywords : $\gamma\delta$ T lymphocytes, CMV, kidney transplantation, mTOR inhibitors

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