Homéostasis and differentiation of CD8 T lymphocytes in the absence of common gamma chain-dependent cytokines

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Hélène Decaluwe

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Homéostasie et différenciation des lymphocytes T CD8$^+$ naïfs et mémoires en absence de cytokines dépendantes de la chaîne γ_c

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Devant le jury composé de :

Pr Pierre-André Cazenave	Président
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Pr Alain Fischer	Examinateur
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Pr James Di Santo	Directeur de thèse

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RÉSUMÉ

Les cytokines de la famille γ_c sont essentielles au développement, à la différenciation thymique et à la survie périphérique des lymphocytes T naïfs. Transmettant leurs signaux par des récepteurs qui ont en commun la chaîne γ_c, les interleukines -2, -7, -15 et -21 sont des facteurs solubles pléiotropes. De par leur redondance lors d'une réponse immunitaire, le rôle individuel des cytokines γ_c dans l'homéostasie des lymphocytes T CD8 et dans la réponse anti-virale n'a été que partiellement élucidé. De plus, l'état actuel des connaissances ne permet pas de savoir avec précision à quel moment de la différenciation et selon quels mécanismes ces cytokines interviennent.

Afin d'évaluer le rôle des cytokines γ_c dans l'homéostasie des lymphocytes T CD8 naïfs, nous avons comparé des cellules monoclonales CD8 issues de souris TCR transgéniques P14 γ_c-compétentes ou γ_c-déficientes. Nous avons montré que les cellules T CD8 naïves $\gamma_c^{-/-}$ ne s'accumulent pas dans les organes lymphoïdes secondaires et que les quelques cellules résiduelles se caractérisent par une petite taille, une diminution de l'expression du CMH de classe I et une augmentation de l'apoptose. Nous avons ensuite corrigé le défaut intrinsèque de survie des cellules T CD8 $\gamma_c^{-/-}$naïves, en surexprimant la molécule humaine Bcl-2, un facteur anti-apoptotique. Cette approche nous a permis de restaurer le nombre de lymphocytes T CD8 naïfs en périphérie, malgré l'absence de chaîne γ_c. Par contre, tout comme ce qui avait été démontré pour les cellules T CD4, l'expression de Bcl-2 ne permet pas de corriger le défaut de taille et de synthèse protéique des cellules γ_c-déficientes. Nous concluons donc que les cytokines γ_c génèrent des signaux Bcl-2-dépendants et Bcl-2-indépendants pour maintenir le phénotype et l'homéostasie des lymphocytes T CD8 naïfs.

Afin de définir l'implication précise des cytokines γ_c au cours de la différenciation des cellules T CD8, nous avons évalué la réponse des cellules T CD8 Bcl-2^+ $\gamma_c^{+/+}$ ou $\gamma_c^{-/-}$ après infection par le virus de la chorioméningite lymphocytaire. De façon tout à fait étonnante, nous avons démontré que de nombreuses étapes de la réponse anti-virale primaire se déroulent normalement en l'absence de chaîne γ_c. En effet, l'expansion clonale, les changements phénotypiques associés à une activation et l'acquisition de fonctions effectrices par les lymphocytes T CD8 γ_c-déficients sont préservés. Par contre, les signaux dépendants de la chaîne γ_c s'avèrent essentiels à la différenciation et la prolifération des effecteurs tardifs ainsi qu'à la génération et le maintien des lymphocytes T CD8 mémoires. Nous proposons donc que les cytokines γ_c-dépendantes ne sont pas indispensables à l'acquisition de fonctions cytotoxiques et à la réponse anti-virale, mais génèrent des signaux Bcl-2-indépendants essentiels à la survie et à la prolifération des cellules T CD8 mémoires.

SUMMARY

Cytokines signalling through receptors sharing the γ_c chain, especially IL-7, are critical for the development and peripheral homeostasis of naïve T cells. Furthermore, IL-2, -7, -15 and -21 are pleiotropic factors that can play complimentary or overlapping roles in T cell homeostasis and immune responses to infection. However, identification of their precise function during an anti-viral immune response has been challenging. Indeed, γ_c deficiency affects not only the survival of naïve T cells but also the function of regulatory T cells, limiting the use of γ_c-deficient mice for assessing T cell immunity. Furthermore, the exact contribution of the γ_c-dependent cytokines in the differentiation of CD8 T cells remains disputed. Indeed, it is unclear at which step of the differentiation process these cytokines impact and what is their importance on the cell-fate decision towards terminal differentiation versus memory generation.

In order to assess the impact of γ_c deficiency on the biology of naïve CD8 T cells, we derived P14 TCR transgenic mice on the recombination-activating gene-2 deficient background with or without γ_c. In this setting, $\gamma_c^{-/-}$ naïve CD8 T cells fail to accumulate in peripheral lymphoid organs and the few remaining cells are characterized by small size, decreased expression of MHC class I proteins and enhanced apoptosis. By over-expressing human Bcl-2, an anti-apoptotic molecule, the number of peripheral naïve CD8 T cells that lack γ_c could be restored. Nevertheless, as described for naïve CD4 T cells, the presence of Bcl-2 could not correct the size and protein synthesis defect of γ_c-deficient CD8 T cells. We conclude that γ_c cytokines provide Bcl-2-dependent as well as Bcl-2-independent signals to maintain the phenotype and homeostasis of the peripheral naïve CD8 T cell pool.

In order to dissect the entire CD8 T cell differentiation program in the absence of γ_c, we compared the response of CD8 T cells from $\gamma_c^{+/+}$ or $\gamma_c^{-/-}$ P14 Bcl-2 mice after challenge with lymphocytic choriomeningitis virus. We demonstrated that although γ_c-dependent signals are dispensable for the initial expansion and the acquisition of cytotoxic functions following antigenic stimulation, they condition the terminal proliferation and differentiation of CD8 effector T cells (i.e.: KLRG1^{high} CD127^{low} short-lived effector T cells) via the transcription factor, T-bet. Moreover, the γ_c-dependent signals that are critical for memory T cell formation are not rescued by Bcl-2 overexpression. Together, our results define the critical stages for γ_c cytokines in the programming of terminal effector CD8 T cells and in the Bcl-2-independent survival and homeostatic proliferation of memory CD8 T cells.

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LIST OF ABBREVIATIONS

A

ACAD : Activated Cell Autonomous Death
AICD : Activation Induced Cell Death
AIRE : Autoimmune Regulator
ALPS : Autoimmune Lymphoproliferative Syndrome
APC : Antigen Presenting Cell

B

Bad : Bcl-2 antagonist of cell death
Bak : Bcl-2 antagonist/killer
Bax : Bcl-2 associated X protein
Bcl : B cell lymphoma
Bcl-X_L : B cell lymphoma extra large
Bid : BH3-interacting domain death agonist
Bim : Bcl-2 interacting mediator of cell death
Blimp-1 : B Lymphocyte-Induced Maturation Protein-1
Brdu : Bromodeoxyuridine

C

CAD : Caspase-Activated DNase
CCL : Chemokine (C-C motif) Ligand
CCR : Chemokine (C-C motif) Receptor
CD : Cluster of Differentiation
cDC : conventional Dentritic Cell
CDK6 : Cyclin-Dependent Kinase 6
CDR : Complementary Determining Region
CFSE : Carboxyfluorescein Succinimidyl Ester
CTL : Cytotoxic T Lymphocyte
CTLA-4 : Cytotoxic T Lymphocyte Associated-4
CXCR : Chemokine (C-X-C motif) Receptor

D

DC : Dendritic Cell
DC-SIGN : DC-Specific ICAM3-Grabbing Non-integrin
DN : Double Negative
DP : Double Positive
DTR : Diptheria Toxin Receptor

E

ERK : Extracellular signal-regulated kinase

F

FADD : Fas-Associated Death Domain
FasL : Fas Ligand

G

GAF : Gamma Activated Factor
γ_c : common gamma chain
GLUT1 : Glucose Transporter 1
GP : Glycoprotein

I

ICAM : Inter-Cellular Adhesion Molecule
ICOS : Inducible Costimulator
ICOSL : Inducible Costimulator Ligand
IL : Interleukin
IFN : Interferon
IFNGR : Interferon gamma receptor
IFNR : Interferon Receptor
ITAM : Immunoreceptor Tyrosine-based Activation Motif
ITIM : Immunoreceptor Tyrosine-based Inhibition Motif

J

Jak : Janus kinase
JNK : JUN amino-terminal Kinase

K

KLRG1 : Killer cell Lectin-like Receptor G1

L

Lck : Leukocyte-specific protein tyrosine kinase
LCMV : Lymphocytic Choriomeningitis Virus
LFA : Leukocyte Function-associated Antigen
LIP : Lymphopenia-Induced Proliferation

M

MAP : Mitogen Activated Protein
Mcl-1 : Myeloid Cell Leukemia sequence 1
MγHV : Murine gamma herpes virus
MHC : Major Histocompatibility Complex
MI : Marilyn
MPEC : Memory Precursor Effector Cell
MTOC : Microtubule Organization Centre
mTOR : mammalian Target Of Rapamycin

N

NFAT : Nuclear Factor of Activated T cell
NFκB : Nuclear Factor kappa B
NK : Natural Killer
NKT : Natural Killer T cell

O

OT : Ovalbumin TCR

P

PAMPs : Pathogen Associated Molecular Patterns
PD-1 : Programmed Death-1
PD-1L : Programmed Death-1 Ligand
PI3K : Phosphoinositide 3 Kinase
PKB : Protein Kinase B
PKC : Protein Kinase C
PLCγ : Phospholipase C gamma
PRR : Pattern Recognition Receptor
Puma : p53-Upregulated Modulator of Apoptosis

R

Rag : Recombination activating gene
RT-PCR : Reverse Transcription-Polymerase Chain Reaction

S

SCID : Severe Combined Immunodeficiency
S1P : Sphingosine-1-Phosphate
SLEC : Short-Lived Effector Cell
SMAC : SupraMolecular Activation Cluster
SP : Single Positive
SPI-6 : Serine Proteinase Inhibitor-6
Stat : Signal transducer and activator of transcription

T

TCR : T Cell Receptor
T_{CM} : Central Memory T cell
T_{EM} : Effector Memory T cell
T_H : T helper
TLR : Toll-Like Receptor
TN : Triple Negative
TNF : Tumor Necrosis Factor
TNFR : Tumor Necrosis Factor Receptor
TRAF : Tumor necrosis factor Receptor Associated Factor
TRAIL : TNF Related Apoptosis-Inducing Ligand

Z

Zap-70 : ζ –chain Associated Protein kinase of 70 kDa

INTRODUCTION

I. Homeostasis of naïve CD8 T cells

A. Overview of thymopoïesis

The thymus supports the development and selection of αβ T cells before their export in the periphery. This differentiation process from T cell progenitors to mature CD8 T cells is tightly regulated and involves numerous transcription factors essential for their development. Upon entry in the thymus, early thymic progenitors are committed to develop into the T cell lineage by the activation of the Notch signalling pathway. Further T cell development will be sustained by Notch signals throughout the journey of the T cell in the thymus (Laky, Fleischacker et al. 2006). Through interactions with specialized stromal and epithelial cells within the thymus, T cell progenitors will go through four distinct phases: generation of double positive thymocytes in the outer cortex, positive selection in the cortex, negative selection in the medulla and finally export of the mature T cells in the periphery (**Figure 1**).

$CD4^+$ $CD8^+$ double positive (DP) thymocytes are the first T cell precursors to express the assembled T cell receptor (TCR) complex at their surface. The TCR rearrangement that precedes is a complex process that provides for the tremendous heterogeneity of the mature T cell pool. $CD3^-$ $CD4^-$ $CD8^-$ triple negative (TN) cells undergo massive proliferation and expansion during this process, through the influence of stem cell factor (ligand of c-kit) and the cytokine interleukin-7 (IL-7), which are simultaneously survival and growth factors. Once established, the pre-TCR and TCR will cooperate to promote the survival and differentiation of the T cell progenitors. However, despite these survival signals, a majority of thymocytes incapable of engaging self-MHC molecules presented by the cortical thymic epithelial cells will not be positively selected and will undergo programmed cell death. Concurrently to the positive selection of potentially useful thymocytes, DPs differentiate into either CD4 helper T cells or CD8 cytotoxic T cells. This precise lineage fate will be determined by the MHC-restriction specificity of their TCR and is likely dependent on the strength of the MHC/TCR interaction (signalling by CD8 and MHC class I is weak and of short duration, thus terminating CD4 transcription) (Singer, Adoro et al. 2008).

Once positively selected, thymocytes must undergo a step of negative selection of potentially self-reactive cells, to prevent possible autoimmunity. Thus, strong MHC/TCR interactions also engage apoptotic signals leading to destruction of auto-reactive clones. Self-peptides being naturally presented by medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) under the control of AIRE (Autoimmune Regulator), negative selection occurs in the medulla, during the DP to single positive (SP) transition. However, some TCR transgenic mice models exhibit negative selection earlier. Early clonal deletion will thus depend on the timing of TCR expression, the cortical and/or medullary site of self-antigen presentation and the affinity of the TCR for its cognate ligand. Therefore, in TCR transgenic mice models, negative selection may proceed throughout the journey of the thymocyte from the cortex to the medulla or be restricted to the medullary region, as it should (Hogquist, Baldwin et al. 2005). In fact, triggering of the TCR will lead to maturation of the thymocytes to become SP and the coordinate upregulation of CC-chemokine receptor 7 (CCR7) and other molecules at the surface of the cell. CCR7 ligands (CCL19 and CCL21) being predominantly produced by mTECs, the thymocytes will be attracted to the medulla. Once in the medulla, SPs that remain after negative selection will mature and be secreted by an active process involving the sphingosine-1-phosphate receptor 1 (SIP_1) in the perivascular space of the thymus (Takahama 2006).

Thymopoïesis is an impressive process, generating an infinite number of naïve T cells with different specificities, but is quite counter-productive, since only 1-3% of thymocytes survive this selective process. In fact, both positive and negative selection will lead to apoptotic cell death of thymocytes (**Figure 2**). Death occurs upon withdrawal of survival signals, being in the thymus mainly stem-cell factor, IL-7, pre-TCR and TCR signals. Cytokine withdrawal will activate BH3-only proteins, in particular Bim, neutralizing the pro-survival Bcl-2 (B cell lymphoma-2) molecules, such as Bcl-2 or Mcl-1. Conversely, loss of pre-TCR and TCR signals decreases the expression of Bcl-X_L and A1, two other pro-survival Bcl-2-family members. Will ensue the activation of Bax and Bak, and the downstream caspase cascade, leading to cell death. Thus, upon cytokine withdrawal, the balance between pro and anti-apoptotic molecules will favor cell death (Opferman and Korsmeyer 2003). The importance of the death receptor apoptotic pathway, as suggested by the study of Fas or Fas Ligand (FasL) deficient mice, appears however to be limited in the thymus but is essential for peripheral tolerance (Palmer 2003). Interestingly, enforced expression of Bcl-2 can prevent death through the intrinsic pathway. In fact, mice deficient for the IL7Rα chain, the common gamma (γ_c) chain and its downstream signalling molecule Jak3 present an

important block in T cell development and thus a severe combined immunodeficiency (SCID) phenotype (Cao, Shores et al. 1995; DiSanto, Muller et al. 1995; von Freeden-Jeffry, Vieira et al. 1995; Baird, Thomis et al. 1998). Enforced expression of Bcl-2 transgene rescues most of the thymocytes from death and restores peripheral T cell numbers (Akashi, Kondo et al. 1997; Kondo, Weissman et al. 1997; Maraskovsky, O'Reilly et al. 1997). Nevertheless, while Bcl-2 can compensate for the loss of IL-7/ γ_c survival signals, it cannot correct for the TN2/TN3 developmental block seen in IL7R$\alpha^{-/-}$ or $\gamma_c^{-/-}$ mice, and thus fails to increase the total thymic cellularity (Rodewald, Waskow et al. 2001).

B. T cell homeostasis

Newly generated mature CD8 T cells exit the thymus to form a pool of long-lived naïve T cells. Once in the periphery, this pool remains stable over time and is regulated by complex homeostatic mechanisms. In fact, the number of naïve T cells is relatively constant, independently of other T cell or non T cell lineage. Thus, the arrival of new thymic emigrants, the survival of naïve CD8 T cells and the slow turnover of those T cells is compensated by apoptotic mechanisms. Once again, as we will see, some of the survival and growth factors involved in thymopoïesis will be involved in the homeostasis of the naïve CD8 T cell pool.

1. T cell survival

Transfer of naïve cells in a MHC class I deficient environment or study of bone marrow chimeric mice lacking MHC class I expression revealed that naïve CD8 T cells require self-MHC contacts to survive (Tanchot, Lemonnier et al. 1997; Takada and Jameson 2009). Furthermore, abrogation of TCR expression or signalling reduced the life span of the naïve CD8 T cell to two to four weeks, thus confirming the absolute requirement for TCR-MHC contacts for the long term survival of the naïve T cell pool (Labrecque, Whitfield et al. 2001; Polic, Kunkel et al. 2001). Interestingly, polyclonal T cells compete with each other for survival signals and thus self-MHC contacts. Thus, transfer of large quantities of naïve T cells will result in short lifespan since the quantity of self-MHC ligands are limited and available only to a finite number of naïve T cells. The requirement for self-MHC contacts depends on the TCR specificity of the naïve cell, established in the thymus. Thus, TCR transgenic cells of different specificities do

not compete with each other. The distinct pro-survival mechanisms induced upon TCR ligation are poorly understood but probably involve typical signalling pathways downstream of the TCR (Seddon, Tomlinson et al. 2003). Constant and low-grade stimulation are most likely required. Recent studies have further revealed the essential role for TCR-mediated calcium influx for the maintenance of CD8 T cell homeostasis (Jha, Badou et al. 2009).

The pro-survival cytokine IL-7 is also essential for the long-term maintenance of the naïve T cell pool (**Figure 3**). Blockage of IL-7 signals with antibodies or adoptive transfers of naïve T cells in IL-7 deficient mice reduces T cell survival (Schluns, Kieper et al. 2000; Tan, Dudl et al. 2001). Conversely, overexpression of IL-7 by an IL-7 transgene increases the size of the naïve T cell pool (Kieper, Tan et al. 2002). Thus, as in the thymus, IL-7 is a vital determinant of long-term maintenance of the mature CD8 T cells. IL-7 is produced constitutively by thymic ephithelial cells and fibroblastic reticular cells (in the secondary lymphoid organs), and in relatively stable amounts, except in situations of severe lymphopenia. Thus regulation of the sensitivity to IL-7 comes from the modulation of the IL7Rα (CD127) chain at the surface of the cell. For the IL-7 signal to occur, IL-7 must bind the γ_c chain, thus activating Jak1 and Jak3, and subsequently Stat5. Stat5 will translocate to the nucleus and induce transcription of pro-survival genes. In the periphery, the dominant pro-survival molecules downstream of IL-7 are Bcl-2 and Mcl-1, Bcl-X_L being not expressed in naïve CD8 T cells. Bcl-2 and Mcl-1 will thus mediate their function by regulating the activity of multiple pro-apoptotic molecules (Bax, Bak, Bad, Bim, Bid, Puma), as it does in the thymus. Opferman et al confirmed the important role for Mcl-1 in naïve T cell homeostasis by demonstrating the severe depletion of naïve T cells in absence of Mcl-1 signals in mature T cells (Opferman, Letai et al. 2003). The crucial role for Bcl-2 was similarly confirmed in Bcl-$2^{-/-}$ mice, with severe reduction in naive CD8 T cell numbers despite near-normal T cell development in the thymus. Interestingly, the recent generation of $Bim^{-/-}$ Bcl-$2^{-/-}$ mice, and restoration of the naïve T cell pool in this context, reveals that death mechanisms regulating T cell homeostasis are mediated primarily through Bim (Wojciechowski, Tripathi et al. 2007).

2. Homeostatic proliferation

Slow turnover of naïve CD8 T cells is essential for the maintenance of the naïve T cell pool. Two types of proliferation have been described: the basal proliferation in

lymphorepleted hosts and the homeostatic proliferation in lymphodepleted hosts (Jameson 2002; Surh and Sprent 2008). In fact, the basal proliferation of the naïve T cell pool reflects the slow turnover of these cells and is minimal when we consider the $CD44^{low}$ population of CD8 T cells. On the other hand, the capacity of the naïve CD8 T cells to replenish the peripheral pool in situations of severe lymphopenia allows for the return to homeostasis. Even though basal proliferation and lymphopenia-induced proliferation (LIP) were often considered to be equivalent, they are not. In fact, the adoptive transfer of naïve CD8 T cells in a lymphopenic environment leads to the acquisition of phenotypic and functional characteristics of memory T cells, which is distinctly different than the maintenance of a naïve T cell pool. However, since the determinants of basal proliferation were initially described in LIP, we will mention some of the cornerstone studies on this subject.

Self-MHC and IL-7 signals are vital for both LIP and basal proliferation of the naïve CD8 T cell pool. Adoptive transfer of naïve CD8 T cells in syngenic hosts made acutely lymphopenic by irradiation confirmed the dependence on these two factors (Ernst, Lee et al. 1999; Goldrath and Bevan 1999; Schluns, Kieper et al. 2000; Tan, Dudl et al. 2001). Thus, LIP is severely diminished in the absence of IL-7 or self-MHC ligands, for both polyclonal and TCR transgenic cells. Furthermore, the increased IL-7 concentration in context of severe lymphopenia favours proliferation and amplifies the weak TCR signals received from contact with self-MHC. Interestingly, some TCR transgenic cells of low affinity, such as CD8 HY cells or CD4 OT-II cells, do not undergo LIP, while high affinity $CD5^{high}$ TCR cells do, suggesting that the TCR signal strength is an important determinant of LIP. The impact of negative regulators of TCR signalling, such as LAG-3, BTLA-4 and SIT, on LIP further demonstrate that it is the combined signal strength of TCR and IL-7 that establish the propensity for LIP. However, typical costimulatory molecules, such as CD28 and CD40, are dispensable for LIP (Surh and Sprent 2008). Since most naïve T cells do not divide under T cell sufficient conditions, it is most likely the increased availability of IL-7 that dictates homeostatic proliferation. This was confirmed by treatment with IL-7/anti-IL-7 antibody complexes, which increased CD8 T cell basal proliferation in lymphorepleted host (Boyman, Ramsey et al. 2008). However, competition for other resources, such as self-MHC contacts, has also been described. Thus, TCR transgenic cells transferred in T cell sufficient hosts of different specificity proliferate, while they do not if transferred in hosts of the same TCR specificity, self-MHC ligands being not available (Kieper, Burghardt et al. 2004). The molecular mechanisms downstream of IL-7 involved in these proliferative functions are not fully understood. Overexpression of Bcl-2 is

insufficient to compensate for the inability of naïve T cells to undergo LIP in IL-7 deficient hosts (Tan, Dudl et al. 2001). However, entry into the cell cycle is promoted by IL-7 induced degradation of the cyclin-dependent kinase inhibitor $P27^{kip1}$ (Li, Jiang et al. 2006). Additional signals are most probably necessary for adequate homeostatic proliferation induced by IL-7.

Other types of homeostatic proliferation have also been described. Cytokine-induced proliferation refers to the capacity of naïve T cells to undergo a very fast rate of homeostatic proliferation in response to high levels of IL-2 and IL-15 and to differentiate into effector and memory cells. Chronic lymphopenia-induced proliferation is the intense proliferation of naïve T cells upon adoptive transfer into immunodeficient mice in response to antigens derived from the commensal flora. Since we are interested by the homeostasis of the naïve CD8 T cell pool in the normal physiologic context of a lymphorepleted host, prior to infection, these issues will not be discussed further.

II. Effector CD8 T cell differentiation

A. Summary of the primary immune response to pathogens

Upon encounter of a pathogen, a naïve CD8 T cell embark on a precise path of differentiation into cytotoxic T lymphocytes (CTLs) and memory T cells. Even though all the parameters impacting on the ability to generate effector functions and sustaining memory development are still unresolved, it appears clear that a naïve CD8 T cell goes through four distinct phases during this primary immune response (**Figure 4**).

The initial phase consists of the activation of the naïve CD8 T cell upon recognition of a pathogen-encoded peptide and depends on short but stable interactions with a mature DC in the peripheral lymphoid organs. The second phase follows with profound expansion of the activated CD8 T cells and differentiation into potent cytotoxic cells. The primed CD8 T cell divides 15 to 20 times and increases its number by 10^4 to 10^5 fold (Butz and Bevan 1998; Murali-Krishna, Altman et al. 1998; Kaech and Ahmed 2001). This activation and clonal expansion leads to major modifications in the CD8 gene expression profile and to differentiation into potent anti-viral effector cells (Oehen and Brduscha-Riem 1998; Kaech, Hemby et al. 2002). The effector CD8 T cells gain access to the inflamed tissues and, via rapid cytokine secretion and granule exocytosis, eliminate the infected cells (Harty, Tvinnereim et al. 2000; Masopust, Vezys et al. 2001; Weninger, Crowley et al. 2001). Follows a phase of significant contraction, where 90-95% of the effector cells are eliminated over the ensuing week (Badovinac, Porter et al. 2002; Bouillet and O'Reilly 2009). Finally, the remaining CD8 T cells establish long-term protection and are maintained by slow basal homeostatic proliferation (Homann, Teyton et al. 2001). The memory cells generated conserve key effector traits and high proliferative potential, thus providing rapid protection against re-infection (Kaech, Wherry et al. 2002).

Multiple parameters impact on the capacity of CD8 T cells to generate potent and functional effector cells. Through the following pages, we will review some of the important aspects involved in CD8 T cell differentiation.

B. Central role of conventional dendritic cells in CD8 T cell differentiation

Conventional dendritic cells (cDCs) have been described as the sentinels of the body and are central in the delivery of antigenic and costimulatory signals to the naïve CD8 T cells. Their migratory faculty allows them to sample the environment for pathogenic stimuli in the peripheral tissues and present these antigens to naïve T cells in the lymph node. As professional antigen-presenting cells (APCs), they drive the activation and proliferation of naïve CD8 T cells. Furthermore, they sense the surrounding inflammation and dictate, to some extent, the fate of the primed T cell (Reis e Sousa 2006).

1. Antigen presentation

Since T cells require the antigen to be processed and presented, the migratory DCs detect and capture a pathogen-derived product, through macropinocytosis, receptor-mediated endocytosis or phagocytosis, and present it to the naïve T cell in the lymph node bound to molecules of the major histocompatibility complex (MHC). The MHC class I molecules present small endogenously synthesized peptides (8-10 amino acids) derived from proteins degraded in the cytosol to CD8 T cells. MHC class II molecules present longer peptides (15 amino acids) derived from exogenous proteins degraded in the endosomal compartment to CD4 T cells (**Figure 5**).

In order to present virally-encoded peptides to naïve CD8 T cells through this direct presentation pathway, migratory or resident DCs need to be directly infected. However, through a process called antigen cross-presentation, the DCs are also able to present exogenous peptides to CD8 T cells. These exogenous antigens come from either infected epithelial cells, migratory DCs or dying cells in the lymph nodes. This cross-presentation phenomenon is thus essential for the generation of anti-viral immunity. However, it has to be noted that this is an oversimplification of antigen presentation, since DCs can present endogenous (that is synthesized by the APC itself) and exogenous (that is synthesized by other cells) peptides to both CD4 and CD8 T cells (Heath and Carbone 2001; Villadangos and Schnorrer 2007; Masson, Mount et al. 2008).

2. DC maturation

The appropriate activation and subsequent differentiation of the CD8 T cell into potent effector cells requires proper maturation of the cDCs (**Figure 6**). In fact, immature cDCs need to sense a "danger signal" in order to embark in a developmental program leading to maturation and efficient antigen presentation. Numerous factors will be recognized as part of this "danger signal" through the binding to specific receptors at the surface of the cDCs (notably pattern recognition receptors, cytokine receptors, Fc receptors, TNFR (tumor necrosis factor receptor) family, sensors for cell death). Bacterial and viral products, as well as inflammatory cytokines and self-molecules, will thus render the cDCs immunogenic. This maturation process is associated with several coordinated events such as loss of endocytic and phagocytic receptors (DEC-205, macrophage mannose receptors, langerine, DC-SIGN, BDCA-2), increased delivery of peptides to the MHC molecules, increased half-life and expression of those MHC molecules, upregulation of numerous costimulatory molecules (CD40, CD80, CD83 and CD86), changes in the expression of chemokine receptors and adhesion molecules and reorganization of the cytoskeleton. The mature cDCs will also secrete numerous cytokines and chemokines, thus recruiting and activating surrounding immune cells. In fact and most importantly, the priming history of the cDC will impact on the activation signals given to the naïve CD8 T cells at the time of antigen presentation (Banchereau, Briere et al. 2000; Guermonprez, Valladeau et al. 2002; Ueno, Klechevsky et al. 2007).

3. Immunological synapse

Hence, it appears that cDCs play a significant role in initiating and controlling the magnitude and the quality of the adaptive immune response. In order to present its peptide and transmit its signals, the mature DC must establish a close contact with the targeted CD8 T cell. This stable interaction is highly organized and is constituted of an ordered distribution of receptors and ligands on each cell, creating a three-dimensional physical area called the immunological synapse (**Figure 7**). Upon contact with a cDC (or a target cell), the T cell microtubule organization centre (MTOC) relocates to an area just underneath the synapse. This reversal of polarity allows for adequate delivery of cytokine vesicles or cytotoxic granules to the cDC or the target cell respectively. In addition to this cytoskeleton rearrangement, the different molecules constituting the synapse arrange themselves in distinct areas within the interface. The TCR complexes

(hundreds to thousands), and its associated costimulatory molecules, locate themselves in the central region of the so-called supra-molecular activation complex (cSMAC), while the adhesion molecules predominate in the peripheral ring, the pSMAC. Bulky molecules, which are not involved in signal transduction, costimulation or adhesion, are located in the distal region (dSMAC) (Monks, Freiberg et al. 1998; Huppa and Davis 2003; Friedl, den Boer et al. 2005).

In order to establish a stable synapse, the CD8 T cells need to bind ten or more MHC-peptide ligands, promoting adequate calcium influx and activation of the NFAT pathway (nuclear factor of activated T cell) (Purbhoo, Irvine et al. 2004). However, the integration of the signals delivered by the DC is not static but rather dynamic, and depends on the antigen dose. In the lymph node, CD8 T cell priming lasts for 1-2 days and involves typically three distinct phases. During the first 8 hours, the CD8 T cell encounters numerous DCs for brief periods of time (less than 10 minutes). Despite the absence of long-lasting synapses, activation is induced and T cells upregulate CD69 and CD44. The second phase lasts 16 hours during which the CD8 T cell establishes long and stable contacts with individual DCs (few hours). The T cells upregulate CD25 and secrete some IL-2 and IFNγ, but don't proliferate yet. Finally, during the last phase, the T cell returns to short-lasting interactions with multiple DCs. They dissociate themselves from the DCs and proliferate vigorously. Indeed, during the course of its activation, the CD8 T cell integrates signals from multiple DCs through different synapses, the type and duration of which impact on its future differentiation (Mempel, Henrickson et al. 2004; Bousso 2008).

C. Three signals to activate a naïve T cell

Multiple factors have been shown to influence the T cell immune response after antigenic presentation by DCs. These include the number of DCs displaying MHC-peptide complexes, the maturation status of the antigen presenting cells, the kinetic of antigenic presentation, the number of naïve T cells displaying the appropriate TCR, the TCR affinities of the responding T cells, the cell intrinsic characteristics of the T cells and the inflammatory environment. The mature DC will thus convey three essential signals to the T cell, programming its activation and differentiation. The signal 1 relies on the TCR engaging with an appropriate peptide-MHC complex on the DC and provides the specificity to the response. By itself, signal 1 may lead to anergy or deletion of the T cell. The signal 2 refers to the compound signals given by the so-

called co-stimulatory molecules. This second signal is essential for the generation of an adaptive immune response. Finally, signal 3 represents inflammatory signals delivered by the DC to the T cell which impact on its differentiation outcome. The different factors influencing these three distinct signals will be reviewed (**Figure 8**).

1. Signal 1: TCR-MHC peptide interaction

Signal 1 refers to the interaction between the TCR and its cognate antigen at the immunological synapse. The αβ TCR recognizes its specific MHC class I-bound peptide through its three highly variable complementary determining region (CDR). The associated CD3 homodimers or heterodimers (constituted of ε, δ, γ, ζ chains) are essential for the surface expression of the αβ heterodimer and for signalling via the receptor. Optimal signal transduction is favoured by clustering with the associated co-receptor chain CD8. Upon peptide recognition and aggregation of the different constituents of the receptor, the ITAM sites of the CD3 chains are phosphorylated by the attached tyrosine kinase Lck. The tyrosine kinase ZAP-70 is then recruited and phosphorylated, activating numerous signalling pathways: the phospholipase c γ (PLCγ) pathway leading to calcium influx and activation of the protein kinase c (PKC) pathway, and the Ras/MAP kinase pathway. Follows the induction of new gene synthesis by activation of major transcription factors, such as NFκB, NFAT and AP-1. These transcription factors will modulate and influence the differentiation outcome of the primed T cell (Janeway, Travers et al. 2001).

The strength of signal 1 is directly influenced by the magnitude and duration of the signal and impacts on the size of the anti-viral CD8 effector T cell response. Several studies have revealed that the magnitude is determined by the antigenic dose, the quantity of MHC/peptide or TCR complexes at the surface of the cells and the TCR affinity. In fact, modulating the density of MHC/peptide complexes on the DCs or infecting with different doses of pathogen influence directly the proliferative potential of the CD8 T cells (Wherry, Puorro et al. 1999; Bullock, Colella et al. 2000; Kaech and Ahmed 2001; Badovinac, Porter et al. 2002; Henrickson, Mempel et al. 2008). Thus, the greater the antigen load, the larger will be the subsequent expansion. Furthermore, the idea that the TCR affinity was essential in determining the strength of signal 1 came from studies reporting that high-affinity CD8 cells had a selective advantage during the immune response over the low-affinity CD8 T cells (Busch and Pamer 1999; Kedl, Rees et al. 2000; Kedl, Schaefer et al. 2002; Zehn, Lee et al. 2009).

As suggested in the previous section, the duration of signal 1 impacts on the expansion and differentiation of CD8 T cells. In fact, numerous papers have revealed that CD8 T cells were "programmed" very early after contact with the antigen-presenting DC. Thus, less than 24 hours is required for a CD8 to embark on a complete program of proliferation and differentiation, without the requirement for further antigenic stimulation (Mercado, Vijh et al. 2000; Kaech and Ahmed 2001; van Stipdonk, Hardenberg et al. 2003). Interestingly, a recent study has proposed that some peptides can shorten the duration required before the establishment of stable interactions and thus increase the expansion and cytotoxic functions of the CD8 T cells (Henrickson, Mempel et al. 2008).

The strength of signal 1 is also correlated to the acquisition of cytokine secretion and cytotoxic functions by the activated CD8 T cells (Wherry, Puorro et al. 1999; Bullock, Colella et al. 2000; Kaech and Ahmed 2001; Tian, Maile et al. 2007; Henrickson, Mempel et al. 2008). This was recently challenged by Bevan's group who showed very elegantly that modulating the antigen presentation duration or the antigen affinity had very little impact on the cytokine secretion or the cytotoxic function of the generated CD8 effector cells, but changed the burst size and the kinetic of expansion (Prlic, Hernandez-Hoyos et al. 2006; Zehn, Lee et al. 2009).

Finally, the magnitude and duration of signal 1 impact on the cell fate of the primed CD8. In fact, blunting the infection with antibiotic treatment, reducing the duration of stimulation with diphtheria toxin treatment of CD11c-DTR mice or increasing intraclonal T cell competition have all been shown to enhance the formation of memory precursor effector cells over the terminally differentiated KLRG1^{high} effector cells (Williams and Bevan 2004; Marzo, Klonowski et al. 2005; Badovinac, Haring et al. 2007; Joshi, Cui et al. 2007). Interestingly, the function of these memory cells did not appear to be altered by the decreased strength of the signal (Williams and Bevan 2004; Prlic, Hernandez-Hoyos et al. 2006; Zehn, Lee et al. 2009).

2. Signal 2: Co-stimulatory signals

The second signal influencing the CD8 immune response is constituted by the integrated signals received from the co-stimulatory molecules at the immunological synapse. Two family of molecules are involved in this signal 2, regulating immunity and tolerance: the B7 family and the TNF family. Since I will not discuss issues

regarding peripheral tolerance, only the molecules involved in the expansion and differentiation of CD8 T cells will be mentioned.

Co-stimulatory molecules are essential for the appropriate expansion and differentiation of CD8 cells, since insufficient TCR signals may lead to anergy and tolerance. The major molecules of the B7 family influencing CD8 differentiation consist of CD80 (B7-1), CD86 (B7-2), inducible co-stimulatory ligand (ICOSL), programmed death ligand 1 and 2 (PD-L1 and 2). TNF family members comprise the CD40L, CD70, 4-1BBL and OX40L molecules. Each of these molecules has its appropriate receptor expressed on the activated T cell, either constitutively or induced by activation. Their ligation can lead to stimulatory or inhibitory signals, thus regulating CD8 T cell expansion. For example, both CD28 and CTLA4 are receptors for CD80 and CD86. While CD28 delivers signals for efficient CD8 expansion, CTLA4 delivers inhibitory signals suppressing their proliferation. In fact, it is the temporal and sequential apparition of these receptors at the surface of the CD8 which dictates the functional outcome of the combined signals received through these B7 and TNF receptor proteins, initially contributing to the production of large populations of effectors and subsequently regulating their expansion (Sharpe and Freeman 2002; Williams and Bevan 2007; Croft 2009).

The co-stimulatory molecules have a positive and synergistic function on TCR-activated CD8 T cells, CD28 being the most potent and most studied of them. Their ligation provides primarily proliferative and survival signals to the CD8 cells, enhancing and sustaining their expansion. They increase the CD8 responsiveness to suboptimal signal 1 and thus decrease its threshold of activation (Kundig, Shahinian et al. 1996; Tan, Whitmire et al. 2000). The molecular mechanisms involved in cell survival are not elucidated for CD8 T cells, but the anti-apoptotic molecules Bcl-2 and Bcl-X_L are involved in the survival signals transmitted to CD4 T cells by co-stimulatory molecules (Rogers, Song et al. 2001). Some of these co-stimulatory molecules contribute also to the generation of appropriate CD8 T cell effector functions (CD28, CD70, 4-1BB) and development of potent secondary responses (CD28, CD70, 4-1BB, ICOS, OX40)(Hendriks, Gravestein et al. 2000; Wallin, Liang et al. 2001; Hendriks, Xiao et al. 2005; Borowski, Boesteanu et al. 2007; Fuse, Zhang et al. 2008).

As stated above, signal 2 molecules can also regulate the appropriateness of the response. This is particularly the case for CTLA-4 and PD-1 that inhibit the TCR- and CD28- mediated signal transduction and arrest the cell cycle. Interestingly, PD-1 is

upregulated in exhausted effector CD8 T cells in the context of chronic viral infections with or without CD4 help, in humans and mice (Barber, Wherry et al. 2006; Day, Kaufmann et al. 2006; Trautmann, Janbazian et al. 2006). Blocking the ligands of PD1 with antibodies restores adequate cytotoxicity and clearance of the pathogen (Barber, Wherry et al. 2006; Trautmann, Janbazian et al. 2006; Ha, Mueller et al. 2008). Thus PD-1 not only regulate the proliferation of activated CD8 cells but also their effector function.

Because these co-stimulatory molecules can have redundant effects, it has sometimes been difficult to establish their individual role in anti-viral immune responses. In fact, CD8 T cell responses to lymphocytic choriomeningitis virus (LCMV) were efficiently induced in mice deficient for CD28, CD27, CD40L or OX-40, while slightly reduced in 4-1BB deficient mice. Other pathogens have yielded different results. In these cases, and as we will see later, other costimulatory or inflammatory pathways can complement the studied defect and promote adequate CD8 effector T cell expansion and differentiation. Most importantly, the strength of signal 1 and the infectious context influence the potential impact of costimulatory signals in CD8 T cell expansion and differentiation. Thus, CD28 has been shown to be fundamental for generation of CTL responses in *Listeria monocytogenes*, vesicular stomatitis virus or influenza virus infections, but as mentioned, is dispensable in LCMV infections (Williams and Bevan 2007; Boesteanu and Katsikis 2009).

3. Signal 3: Pro-inflammatory cytokines

Besides its role on DC maturation and antigen presentation, and its ability to augment the expression of costimulatory molecules, the environmental milieu has been shown to directly impact on the CD8 T cell expansion and differentiation after an infectious challenge. In fact, numerous studies and reviews have reported in the last few years the importance of these pro-inflammatory cytokines in the acquisition of adequate cytotoxic functions and the transition from effector to memory cells (Haring, Badovinac et al. 2006; Kaech and Wherry 2007; Williams and Bevan 2007; Harty and Badovinac 2008; Joshi and Kaech 2008; D'Cruz, Rubinstein et al. 2009). To date, three major cytokines are known to participate in this signal 3: interleukin-12 (IL-12), type 1 interferons (IFNα/β) and IFNγ.

Depending on the infectious agent studied, IL-12, type 1 IFNs and IFNγ have all been shown to directly support the expansion and function of effector CD8 T cells by increasing their proliferation and survival, and promoting the development of potent cytotoxicity. In this case, survival signals occur through the upregulation of the anti-apoptotic molecule Bcl-3 (Valenzuela, Hammerbeck et al. 2005). The expression of their receptor is modulated throughout the infection. Thus, these cytokine-driven signals might prevail at different moments of the immune response, regulating the expansion and differentiation of CD8 T cells. While IL-12 signals appear to be important throughout the expansion phase, the co-stimulatory IFNγ signals are essential in the first day following antigenic challenge (Whitmire, Tan et al. 2005). Moreover, type 1 IFN receptors (IFNR) are downregulated just after infection but are reexpressed rapidly (Haring, Badovinac et al. 2006).

By studying receptor knockout mice to these different molecules, an essential role for those cytokines in the differentiation of CD8 effector T cells was revealed. Interestingly, once again, it appears that these cytokines have redundant functions in mice and that the infectious agent used determines the dependency on one or the other cytokine. After LCMV infection, IFNα/βR$^{-/-}$ effector CD8 T cells are severely impaired in their survival potential and thus total expansion. Furthermore, while cytokine secretion is preserved, granzyme B levels and viral clearance are severely diminished (Cousens, Peterson et al. 1999; Kolumam, Thomas et al. 2005; Aichele, Unsoeld et al. 2006; Thompson, Kolumam et al. 2006). On the contrary, IFNγR1$^{-/-}$ mice show moderate expansion and IL12p35$^{-/-}$ mice exhibit normal expansion upon LCMV infection (Cousens, Peterson et al. 1999; Whitmire, Tan et al. 2005). In contrast, *Listeria monocytogenes* infections are very sensitive to IL-12 signals, but are not affected by blockage of the type 1 IFNs or IFNγ pathways (Badovinac, Tvinnereim et al. 2000; Thompson, Kolumam et al. 2006). Interestingly, LCMV is an important producer of type 1 IFNs but produces virtually no IL-12, while *Listeria monocytogenes* infections produce primarily IL-12 and IFNγ. Once more, the response to a pathogen is translated into a unique pattern of inflammation, and this inflammation regulates differently the priming and differentiation of CD8 T cells. As we will see later, this inflammatory context, and thus the signal 3 cytokines involved, influences also the cell fate decisions, the contraction phase and the generation of memory cells.

D. CD4 help in CD8 effector differentiation

The role of CD4 help in activation and differentiation of CD8 T cells is still an area of intense debate. In the last few years, numerous papers have challenged the classical model of CD4 help. These studies have now confirmed that CD4 help is essential in the programming and/or maintenance of potent secondary immune responses - the mechanisms involved in this particular aspect will be analyzed in the next chapter. However, the role for CD4 T cells in the expansion and the differentiation of CD8 effector T cells remains controversial (Bevan 2004).

Classically, the role for CD4 help has been thought to come from their ability to license the DCs into potent antigen presenting cells (**Figure 9**). In fact, after antigen uptake and processing, the DC presents major histocompatibility complex (MHC) class II bound peptide to the CD4 T cell, which in turn upregulates CD40L expression. Through interaction with CD40 on the mature DC, the CD4 T cell potentiates the costimulatory signals given by the B7 and TNF family molecules and increases the secretion of IL-12. Interestingly, the CD4 cell per se is not required to license the DC, since treatment with an agonist antibody for CD40 could replace the requirement for CD4 T cells (Bennett, Carbone et al. 1998; Schoenberger, Toes et al. 1998). In this model, the CD4 help leads to the creation of three cell clusters, enhancing the proximity between the cellular partners and the activation of the CD8 T cell (Beuneu, Garcia et al. 2006). This clustering is promoted by the upregulation of CCR5 on the naïve T cells and presence of CCL3/CCL4 at the site of CD4 T cell /DC interactions (Castellino, Huang et al. 2006). It is thus thought that both T cells recognize the antigenic epitope on the same DC, but that the CD8 T cell receives its signals through direct interaction with the DC, and not the CD4 T cell, at least during the initial phase of the response (Beuneu, Garcia et al. 2006). Furthermore, some authors suggest that non-cognate antigens might also deliver help signals to the DC, promoting further activation of the CD8 cell (Fernando, Khammanivong et al. 2002).

This CD4 help model was initially thought to be true for all antigens, but numerous studies done with pathogens, and thus in the context of high pro-inflammatory signals, prompted revision of the model (**Figure 9**). In fact, in LCMV or *Listeria* infections for example, expansion and potent effector functions could be generated in the absence of CD4 T cells or MHC class II molecules. It was thus concluded that certain infectious agents were able to circumvent the need for CD4 help

through the stimulation of PRRs on the DC or the release of inflammatory cytokines, which promoted the maturation of the DCs. This was confirmed by elegant studies showing a role for type 1 IFNs in the appropriate generation of unhelped CD8 effector T cells (Le Bon, Etchart et al. 2003). Furthermore, Johnson et al. demonstrated the role for the upregulation of CD40L on the TLR3/9-activated DCs in the priming of CD8 T cells, demonstrating another potential mechanism behind the absence of requirement for help in infectious situations (Johnson, Zhan et al. 2009). Once again, as we saw earlier for the other stimulatory signals, multiple redundant pathways collaborate to allow for the generation of potent and numerous cytotoxic CD8 T cells.

The world of CD4 help was once again challenged when some authors proposed that CD4 help was in fact dispensable for the expansion and acquisition of effector functions by most of the former T_H dependent antigens (cellular antigens). In fact, by using tetramer and *ex vivo* intracellular cytokine stainings, they were able to confront the previous data and show that it was the requirement for prolonged *in vitro* activation (6 days) that probably lead to false conclusions. These former techniques were in fact highlighting the abnormal secondary responses seen in unhelped CD8 T cells, but not the actual primary responses. After these studies, it was concluded that the primary immune responses to most antigens were dispensable of CD4 help, but that generation and maintenance of memory cells were dependent on it (Bourgeois, Rocha et al. 2002; Fernando, Khammanivong et al. 2002; Janssen, Lemmens et al. 2003). However, it is still possible that the priming protocols used in those studies provide some kind of danger signals sensed by the DC allowing for their proper maturation, and thus help-independent activation. Furthermore, it appears that the peak expansion and the cytotoxic functions of these primary CD8 T cells are often diminished. Finally, other approaches confirmed the absolute requirement for CD4 help in response to cellular antigen (Wang and Livingstone 2003). Moreover, herpes simplex infection has recently been shown to depend on CD4 help for the generation of potent effector responses (Johnson, Zhan et al. 2009; Rajasagi, Kassim et al. 2009). It thus appears that, depending on the situation, CD4 help might or might not be essential to the primary immune response to the so-called T_H dependent and independent antigens. However, it is likely that CD4 cells are contributors to the primary CD8 immune response, by promoting the recruitment of cognate CD8 cells around mature DCs and delivering differentiation signals to them.

E. γ_c-dependent cytokines in CD8 T cells expansion and differentiation

The contribution of γ_c dependent cytokines in the survival and homeostasis of naïve and memory CD8 T cells has been clearly demonstrated, and is discussed in other sections. The differential expression of the different cytokine-receptor subunits during the immune response suggest that some of these cytokines might have a role in the proliferation of CD8 T cells (**Figure 10**). In this section, we will review the known *in vivo* functions of γ_c dependent cytokines during this precise expansion and differentiation period. We will later come back on the impact of these cytokines in the generation and maintenance of memory cells.

1. IL-2

Activated CD4 T cells, CD8 T cells and DCs produce IL-2. Being the major cytokine produced by CD4 T cells, IL-2 has been proposed as a mediator for CD4 help in some models, as discussed in the previous section. IL-2 ligation with its heterotrimeric receptor, composed of the IL-2Rα (CD25), IL2Rβ (CD122) and IL-2Rγ (CD132/γ_c) chain, induces several signal transduction pathways, including activation of the Jak1-Jak3/Stat5 pathway, phosphorylation of Lck, stimulation of the PI3K/AKT pathway and the Ras/MAP kinase pathway. TCR stimulation leads to extremely rapid expression of CD25 at the surface of the CD8 T cells, and maintenance for 72 hours. *In vitro*, IL-2 induces strong proliferative signals to CD8 T cells. It is also essential for the development of effector functions, such as IFNγ secretion and granzyme B-dependent CTL killing of target cells (Malek and Bayer 2004).

In vivo, the importance of IL-2 is more modest. It appears to be necessary for maximal generation of effector T cells at the peak of the response, in an antigen-dependent manner (Cousens, Orange et al. 1995; Williams, Tyznik et al. 2006). However, IL-2Rα$^{-/-}$ mice have only 2-fold less effector CD8 T cells than their wild type counterparts, demonstrating that this cytokine is not essential for CD8 T cell expansion (Williams, Tyznik et al. 2006). In fact, IL-2 requirement is minimal during the first 48 hours and is principally required to sustain the proliferation of CD8 T cells in non-lymphoid organs (D'Souza, Schluns et al. 2002; D'Souza and Lefrancois 2003). Furthermore, initial reports on the effector functions generated in the absence of IL-2

signals are conflicting, with controlled or uncontrolled LCMV infections and potent rejection of allografts (Kundig, Schorle et al. 1993; Cousens, Orange et al. 1995; Steiger, Nickerson et al. 1995). However, CTL cytotoxicity assays were diminished - although not abrogated- in the absence of IL-2 signals. Interestingly, IL-2Rα$^{-/-}$ CD8 effector T cells express more CD62L and CD127 at their surface and secrete more IL-2 than their IL-2Rα$^{+/+}$ counterparts, demonstrating that IL-2 might be essential for the complete differentiation of effector T cells (Williams, Tyznik et al. 2006). It would have been interesting to test the functions of those cells in the context of acute LCMV infection, since the initial reports were done in mice with defects in regulatory T cell numbers and functions, thus perturbing our interpretation of the data. Of note, recent papers propose an essential role for IL-2, and in particular Stat5 signals, in the expansion and differentiation of CD8 T cells in the absence of potent TCR activation (Verdeil, Chaix et al. 2006; Verdeil, Puthier et al. 2006; Kamimura and Bevan 2007). This confirms the role for IL-2 in the priming and costimulation of effector CD8 T cells.

2. IL-15

IL-15 secretion is induced primarily in response to TLR or IFN signals by activated DCs and macrophages. It is membrane-bound and presented in *trans* by the IL15Rα chain, which will bind to the IL2Rβ and IL2Rγ chain, common to the IL-2 receptor (Dubois, Mariner et al. 2002). The signalling cascade will thus be common for both cytokines. However, because of its *trans* presentation, IL-15 requires cell-cell contact at the immunological synapse to deliver its signals (**Figure 11**). Despite their common receptor subunits and signal transduction pathways, IL-2 and IL-15 have distinct functions. *In vitro*, like other γ_c-dependent cytokines, IL-15 promotes expansion of the CD44^{high} CD8 population (Vella, Dow et al. 1998; Manjunath, Shankar et al. 2001; Judge, Zhang et al. 2002). However, despite potent IFNγ secretion, IL-15 primed CD8 T cells are poor killers (Manjunath, Shankar et al. 2001).

In vivo, the requirement for IL-15 in the expansion and effector function of CD8 T cells is controversial. In response to LCMV or *Listeria* infection, IL-15$^{-/-}$ and IL15Rα$^{-/-}$ mice generate a robust primary response, with efficient clearance of the pathogen (Becker, Wherry et al. 2002; Yajima, Nishimura et al. 2005; Yajima, Yoshihara et al. 2006). The expansion of effector T cells to major dominant epitopes is similar to wild type mice, except for the GP_{33-41}^{+} population, and the surface expression of relevant activation markers is identical (Becker, Wherry et al. 2002). In contrast, IL-15 signals

appear to be important in the context of vaccinia virus infection or peptide-based vaccination. In the vaccinia virus setting, IL-15 seems to potentiate the proliferation of CD8 T cells during the last days of the expansion phase (Schluns, Williams et al. 2002). In peptide-loaded DC vaccination, treatment with IL-15 promotes CD8 T cell expansion and cytotoxic functions (Rubinstein, Kadima et al. 2002). Once again, depending of the infectious context, it appears that this second important γ_c cytokine may potentiate proliferative signals received by the CD8 T cells, especially during the last days of the expansion phase, but may not be essential for the differentiation of cytotoxic CD8 T cells.

3. IL-21

Activated CD4 T cells and NKT cells are the principal producers of IL-21, the last member of the γ_c family of cytokines. Its receptor, composed of the IL21Rα and the γ_c chain, is expressed constitutively on multiple cells, including CD8 T cells, and upregulated in response to TCR or IL-21 signals. Transduction through NFAT is essential for its synthesis, and activation of Stat3 for its function. Granzyme A, granzyme B and Bcl-3 are some of the IL-21 target genes. In T cell immunology, IL-21 has been mainly described for its role in Th17 cell development and function. *In vitro*, IL-21 appears to potentiate the proliferative functions of other γ_c-dependent cytokines, in the presence or absence of TCR signals (Parrish-Novak, Dillon et al. 2000; Zeng, Spolski et al. 2005). Furthermore, it increases cytokine secretion and cytotoxic functions of activated CD8 T cells (Liu, Lizee et al. 2007). Its costimulatory function might be linked to its ability to prevent the downregulation of CD62L and CD28 upon IL-15 driven signals, at least in humans (Alves, Arosa et al. 2005).

In vivo, the importance of IL-21 varies depending on the model studied. In response to vaccinia virus infection, IL21Rα$^{-/-}$ CD8 T cells proliferate less than controls, and have reduced cytotoxic functions (Zeng, Spolski et al. 2005). In contrast, IL-21 is not required for the expansion and function of CD8 T cells in acute LCMV infection, although IL-21 expressing cells have a slight growth advantage (Elsaesser, Sauer et al. 2009). The role of this cytokine as a possible mediator of CD4 help in CD8 T cell responses has been recently investigated, especially in the context of chronic LCMV infection. In a recent study, Elsaesser et al suggest that the absence of IL-21 signalling prevents the downregulation of IL-2 secretion by CD4 cells, which will lead to exhaustion and abnormal viral clearance in the context of chronic LCMV infection

(Elsaesser, Sauer et al. 2009). Furthermore, IL-21 treatment of $CD4^{-/-}$ mice infected with LCMV Clone 13 will increase the functionality of the exhausted CD8 T cells and decrease the viral titers (Yi, Du et al. 2009). Finally, IL-21 appears to have a prominent role in primary anti-tumor CD8 responses, especially in conjunction with IL-15 (Zeng, Spolski et al. 2005). It promotes the development of potent anti-tumoral CD8 cells, by increasing their proliferation, expansion and cytotoxic functions (Moroz, Eppolito et al. 2004).

4. IL-7

Lastly, multiple studies have looked at the role of IL-7 in the primary expansion of effector CD8 T cells. As suggested by the downregulation of IL7Rα upon T cell activation, IL-7 has not been shown to be essential for the expansion and differentiation of primary effector CD8 T cells in numerous infectious models (LCMV, vesicular stomatitis virus, *Listeria* infections) (Schluns, Kieper et al. 2000; Klonowski, Williams et al. 2006; Osborne, Dhanji et al. 2007; Nanjappa, Walent et al. 2008). Furthermore, overexpression of IL-7 or IL-7 treatment does not support the proliferation of $KLRG1^{high}$ effector CD8 T cells (Hand, Morre et al. 2007; Rubinstein, Lind et al. 2008).

F. CD8 effector functions

The ultimate goal of CD8 T cell activation and proliferation is its differentiation into potent effector T cells, capable of rapid pathogen clearance. This effector T cell will eliminate infected target cells via two independent mechanisms, granule exocytosis and death-receptor-induced apoptosis. These two major pathways are important for both immune surveillance and peripheral homeostasis. Furthermore, CD8 effector T cells release TNFα and IFNγ, essential inflammatory cytokines involved in T cell-mediated immunity. However, it has to be noted that NK cells and CD4 T cells share with CD8 T cells the same effector mechanisms. In fact, because of the non-exclusive expression of these molecules and their pleiotropic effect, understanding their contribution in CD8 T cell resistance to infection is challenging. Furthermore, the relevance of each of these factors may differ between infectious agents. It is likely the combined involvement of these mechanisms that contribute to pathogen clearance and immune protection (Harty, Tvinnereim et al. 2000).

1. Cytokine secretion

The major cytokine produced by the effector T cells is IFNγ. Upon TCR and/or IL-12 signalling, the CD8 T cells remodel the chromatin within the *IFNγ* locus and up-regulate IFNγ-promoting transcription factors, allowing for the synthesis and release of important amounts of IFNγ. Among numerous transcription factors involved in IFNγ production, two have been shown to be regulating simultaneously the differentiation and IFNγ production of CD8 T cells: the T-box family members T-bet and eomesodermin (Pearce, Mullen et al. 2003; Sullivan, Juedes et al. 2003; Intlekofer, Takemoto et al. 2005). Naïve CD8 T cells are committed to become IFNγ producers by the constitutive expression of eomesodermin, and *eomes* was though to be sufficient for IFNγ production. However, recent papers confirmed the dependency on T-bet for the production of IFNγ by CD8 T cells, as seen in CD4 T cells (Mayer, Mohrs et al. 2008). Interestingly, IFNγ regulates the transcription of T-bet in a positive feedback loop promoting CD8 differentiation. As we will see later, these transcription factors also influence the lineage choice of the activated T cells.

IFNγ binds to its ubiquitous receptor composed of two ligand-binding chains (IFNGR1) and two signal-transducing chains (IFNGR2). Ligation of IFNγ leads to the activation of JAK 1 and 2 and subsequent phosphorylation of Stat-1. Homodimerization of Stat-1 will form the Gamma Activated Factor (GAF) complex that will translocate to the nucleus to induce the transcription of target genes (Schroder, Hertzog et al. 2004). IFNγ regulates more than 200 genes and will thus have multiple functions in the immune system. In response to infectious challenge, IFNγ contributes to pathogen clearance directly and indirectly, through activation of the innate immune system. Indeed, IFNγ is capable of direct inhibition of viral replication by activation of kinases and deaminases interrupting viral synthesis. It also contributes to macrophage activation and survival, increasing their phagocytosis and killing abilities, essential for the elimination of infected cells. Moreover, IFNγ promotes class I and II antigen presentation, thus amplifying the priming of CD4 and CD8 T cells. Finally, it enhances recruitment of monocytes and lymphocytes at the site of inflammation by promoting the release of numerous chemokines and adhesion molecules.

The importance of this cytokine in the resistance to infection has been highlighted by the study of IFNγ$^{-/-}$ and IFNGR1$^{-/-}$ mice and the discovery of human genetic mutations of the IL-12/IFNγ pathway. While IFNγ$^{-/-}$ and IFNGR1$^{-/-}$ mice are

highly sensitive to infections by multiple intracellular pathogens, humans with Mendelian Susceptibility to Mycobacterial Diseases are susceptible to environmental mycobacteria (Filipe-Santos, Bustamante et al. 2006; Schoenborn and Wilson 2007). Interestingly, IFNγ signals are essential for adequate response to chronic, but not acute, LCMV infections. Furthermore, IFNγ secreted by CD8 T cells, but not innate cells, is dispensable for clearance of *Listeria* infections (Harty, Tvinnereim et al. 2000).

The second important cytokine produced by effector CD8 T cells is TNFα. This cytokine is primarily active as trimers and binds to either TNFR1 or TNFR2, receptors belonging to the TNF receptor super family. TNFR1 is ubiquitously expressed and TNFR2 is mainly expressed by hematopoietic and endothelial cells. In response to TNF ligation, the TNFR binds to one or more TNFR-associated factors (TRAFs) that subsequently leads to activation of the transcription factors NFκB and MAP kinases (ERK, p38, JNK) (Aggarwal 2003). The biological actions of TNFα are thus diverse and involve the regulation of both cellular proliferation and cellular apoptosis. In immune responses against infections, TNFα appears to mediate its positive action via three distinct mechanisms. It synergizes with IFNγ to stimulate macrophage activation and their bactericidal and parasiticidal functions. It also upregulates adhesion molecule expression on endothelial cells, promoting recruitment of innate cells at the site of infection. Lastly, through its intracytoplasmic death domain, TNFα induces caspase-8 dependent apoptosis of infected target cells.

Once again, the importance of this cytokine in resistance to infections differs between pathogens. While it is dispensable during LCMV infections, TNFα is essential for secondary immune protection against *Listeria* infections. Furthermore, in humans, the link between anti-TNFα treatments and the resurgence of mycobacterial tuberculosis reveals the absolute requirement for this cytokine in some infections (Bruns, Meinken et al. 2009). However, TNFα may also have significant side effects following severe infections, as exemplified by its adverse role in cerebral malaria and sepsis (Harty, Tvinnereim et al. 2000; Pfeffer 2003).

2. Granule exocytosis

T cell mediated cytotoxicity is essential for the elimination of infected target cell and ultimate pathogen clearance. Two distinct and complementary pathways exist to achieve target cell lysis: the granule exocytosis pathway and the death-receptor

pathway, the former one being the most important for the elimination of infected cells. Both of these pathways are activated in response to signals from the TCR and stimulate the caspase cascade in the target cell, leading to apoptotic cell death.

Efficient lysis by the granule exocytosis pathway requires the combined action of the pore-forming protein perforin and the cytolytic molecules granzymes. Upon recognition of pathogen-derived peptides at the surface of an infected cell, the CTL establishes a stable but transient immunological synapse. This synapse will allow the targeted release of the cytotoxic granules in the infected cell. The granules accumulate in close proximity of the MTOC and are shunted through a specialized region within the cSMAC. However, recent data suggest that granule polarization and cytolysis may occur without the formation of a cSMAC (O'Keefe and Gajewski 2005). Interestingly, the establishment of a cytotoxic immune synapse requires the engagement of only three to ten peptide-MHC complexes. The synapse duration is short, with calcium influx of only 2-20 minutes, and cells detach rapidly thereafter. Moreover, effector CTLs can form simultaneously many synapses with several target cells, allowing rapid elimination of the pathogen (Huppa and Davis 2003).

The cytotoxic granules are specialized secretory lysosomes constituted of perforin and granzymes. Their number increases during the differentiation of CTLs, upon TCR triggering and γ_c-dependent cytokine stimulation (IL-2, IL-15 and IL-21), at least in NK cells (Glimcher, Townsend et al. 2004). Recent studies have suggested that the two T-box transcription factors T-bet and eomesodermin and the B lymphocyte-induced maturation protein (Blimp-1) regulate the expression of both perforin and granzyme B (Pearce, Mullen et al. 2003; Sullivan, Juedes et al. 2003; Kallies, Xin et al. 2009). Bcl-6, a transcription repressor, might also be involved in the suppression of granzyme B expression in naïve and memory cells (Yoshida, Sakamoto et al. 2006). The low pH and inhibitory functions of cathepsin B and calreticulin protects the CTL from its own destruction (Lieberman 2003).

Granzymes' capacity to induce target-cell death is absolutely dependent on perforin, as highlighted by the severe susceptibility to LCMV infections in perforin knockout mice (Kagi, Ledermann et al. 1994; Walsh, Matloubian et al. 1994). However, the precise mechanisms involved are not fully understood (**Figure 12**). The first model relies on the membranolytic properties of perforin. In this model, the polymerization of perforin creates pores in the target-cell membrane, allowing the creation of an osmotic gradient leading to target cell lysis. Through these pores,

granzymes can also gain entry into the cytosol, leading to target cell death through caspase dependent and independent pathways. The second model proposes that granzymes may enter the cell in endosomes, either by binding to the cell surface electrostatically or by its ligation to the mannose-6-phosphate receptor. The presence of perforin in the same endosome is then essential for granzymes' release into the target-cell cytosol.

Among the granzymes present in the granules, granzyme B has the strongest pro-apoptotic function. It can cleave its substrates after aspartate residues, like caspases, and thus activate caspase-3 to induce apoptosis associated with rapid DNA fragmentation. However, the principal apoptotic mechanism used by granzyme B is target cell killing through caspase-independent pathways. This second pathway relies on the cleavage of Bid (a BH3 interacting death domain agonist) and the induction of mitochondrial damage and cytochrome c release. Caspase-activated DNAse (CAD) can also be directly activated by granzyme B, amplifying DNA fragmentation. Conversely, Granzyme A activates a slower mechanism of caspase-independent apoptosis through the targeting of an endoplasmic reticulum-associated complex, SET. In fact, these two granzymes act independently and synergistically to induce apoptosis. Other granzymes are also present in the cytotoxic granules but their exact role in apoptosis is less well defined (Lieberman 2003).

As described earlier for other effector molecules, the relative relevance of the granule exocytosis pathway depends on the infectious agent encountered, the site and dose of infection and the immunity of the host. In fact, in certain circumstances, other cytotoxic pathways, in particular the Fas-dependent pathway, can complement or even surpass the granule exocytosis pathway. For example, while perforin and granzymes are essential in LCMV infections, they are dispensable in *Listeria* infections (Harty, Tvinnereim et al. 2000). Finally, besides its fundamental role in pathogen clearance, the granule-dependent cytotoxic pathway is critical in controlling the immune homeostasis after an infection. Indeed, because of their inability to clear the virus, perforin$^{-/-}$ mice develop a dysregulated response with sustained CD8 T cell activation, leading to important cytokine release and development of hemophagocytic lymphohistiocytosis syndrome (Jordan, Hildeman et al. 2004). Similar syndromes have been described in humans with genetic mutations in perforin or molecules involved in the polarization, docking, priming, fusion and exocytosis of the granules at the immunological synapse (Menasche, Feldmann et al. 2005).

3. Death-receptor induced apoptosis

Death-receptor mediated cytotoxicity is the second pathway involved in pathogen clearance. While it is the most important killing mechanism of CD4 T cells, it complements the granule exocytosis pathway in CD8 T cells. This pathway is initiated by the ligation of the death-receptors expressed at the surface of the antigen-specific target cell (**Figure 13**). Three members of the TNFR superfamily can induce rapid target-cell apoptosis once ligated: Fas (CD95), TRAIL receptor (TNF-related apoptosis-inducing ligand) and TNF receptor. Immediately after activation, the adapter molecule FADD (Fas-associated death domain) binds to the death domain of the receptor and recruits pro-caspase-8. Once activated, caspase-8 initiates the caspase cascade resulting in apoptotic death of the target cell (Brunner, Wasem et al. 2003). Interestingly, despite the presence of soluble FasL, it was recently confirmed that the membrane-bound FasL is the only form capable of target cell lysis. Conversely, the soluble FasL form seems to have deleterious effects, potentiating the development of autoimmune diseases and tumors (O' Reilly, Tai et al. 2009).

Once again, this pathway is highly regulated. Upon TCR stimulation, multiple transcription factors involved in the regulation of FasL expression are activated. NFκB, AP1, eGR2/EGR3, NFATs have all been described to interact directly with promoter regions of FasL or to modulate its transcription. Recently, CD28 costimulation and IL-2 stimulation have also been shown to influence the transcription of FasL (Glimcher, Townsend et al. 2004). As described earlier for the granule exocytosis pathway, this apoptotic pathway is not only involved in T cell cytotoxic functions but is also essential for peripheral tolerance. Two natural mice mutants with severe lymphoproliferative diseases, the *gld* and *lpr* mice, and human genetic defects in patients with ALPS (autoimmune lymphoproliferative syndrome) highlight the importance of FasL in the regulation of immune homeostasis (Rieux-Laucat, Fischer et al. 2003).

In the immune response to infectious, it is interesting to note that CD8 T cells can coexpress simultaneously FasL and cytolytic granule proteins. However, it is likely that CD8 T cells preferentially use one cytotoxic effector mechanism over the other. This choice might be directed by the site of infection, the type of infectious agent, the acuteness or chronicity of the infection, or the degree of prior activation. This time, both acute LCMV or *Listeria* infections seem to be cleared without the help of FasL-mediated apoptosis (Harty, Tvinnereim et al. 2000). However, a chronic infection to

mouse γ herpes virus (MγHV) for example requires the Fas pathway for its clearance. It is likely the persistent TCR stimulation present in chronic infections that causes the dependency on Fas-mediated signals (Hughes, Belz et al. 2008).

G. Migration to peripheral tissues

T cell migration between sites of priming and inflamed tissues is a tightly regulated process. Selectins, chemokine receptors and integrins are essential to guide the movements of the CD8 T cell in and out of the lymph node and towards the infected sites. Selectins enable T cells to adhere and roll on endothelial venules, while chemokine receptors mediate their firm adhesion by activating integrins, essential for T cell arrest and transmigration. Expression of those different homing receptors is modulated during the course of the immune response and directs the trafficking of CD8 T cells. Interestingly, this process increases the heterogeneity of the effector T cell pool and impacts on the localisation, retention and function of the cells (Bromley, Mempel et al. 2008; Forster, Davalos-Misslitz et al. 2008).

Naïve T cells express three principal homing receptors at their surface, L-selectin (CD62L), CCR7 and LFA-1, allowing for retention and movements inside a lymph node, and circulation through the secondary lymphoid organs of the body. CD62L binds peripheral node addressins on high endothelial venules, while CCR7 interacts with CCL19 and CCL21, its two displayed ligands. Firmer adhesion is provided by attachment of LFA-1, expressed at low levels, to ICAM-1. In fact, naïve T cell motility, and thus localisation in the lymph node, is strictly dependent on CCR7 binding to CCL19 or CCL21, chemokines produced by endothelial cells and fibroblastic reticular cells (Forster, Schubel et al. 1999; Okada and Cyster 2007). Furthermore, CCR7 allows re-entry of the T cell through draining lymphatics (Bromley, Thomas et al. 2005; Debes, Arnold et al. 2005). As we will see later, CD62L and CCR7 molecules are reexpressed in some memory cells and might influence the proliferation capacity, effector function and localization of those cells.

Upon inflammatory signals and fever, CCL21 and ICAM-1 expression in the lymph node is potentiated, allowing for efficient T cell recruitment and trafficking (Chen, Fisher et al. 2006). Moreover, after TCR stimulation, CCR7 and CD62L cell surface expression is transiently maintained, allowing for efficient T cell activation. Soon after, the expression of these two molecules is lost, and activated T cells

upregulate the expression of various combinations of adhesion and chemokine receptors as part of their differentiation program. Through upregulation of CCR5 and CCR2 for example, effector CD8 T cells will have a greater capacity to migrate to inflamed tissues. Interestingly, the site of entry of the pathogen influences the homing potential of the CD8 T cells, through DC-mediated signals. Intracutaneous injection of bone marrow-derived DC leads to increased E-selectin ligand expression, while intraperitoneal injection induces the gut-homing integrin $\alpha 4\beta 7$ expression on CD8 T cells (Dudda, Simon et al. 2004). This was confirmed by a very nice study looking at the expression of selectins and integrins on effector CD8 T cells after implantation of tumor cells subcutaneously, intraperitoneally or intracranially (Calzascia, Masson et al. 2005). Chemokines and their receptors are also critical for directing the homing of lymphocytes to specific tissues. For example, CCR9 defines a subset of lymphocytes with tropism for the small intestine, whereas CCR4 and CCR10 direct skin-tropic T cell trafficking, at least in CD4 T cells (Agace 2006).

III. Memory CD8 T cell differentiation

Development of protective populations of memory cells is the hallmark of adaptive immunity. These memory cells will have specific characteristics allowing them to respond quickly and efficiently to re-infection and to be maintained for an extended period of time. The generation and maintenance of memory cells depend on multiple factors, which will be reviewed in the following pages.

A. Contraction

The abrupt loss of effector cells at the conclusion of the immune response is essential to prevent the persistence of dominant clones and allows for the reestablishment of peripheral homeostasis. From this contraction, few cells will be preserved to generate a new memory cell pool. Multiple control mechanisms are thus used to regulate the contraction of the effector T cell response and select for the memory cell precursors. 90-95% of the effector cells that are no longer needed at the end of the expansion phase will undergo apoptosis through two principal apoptotic pathways: the extrinsic or death receptor pathway or the intrinsic or mitochondrial pathway (Krammer, Arnold et al. 2007; Bouillet and O'Reilly 2009) (**Figure 14**). Some characteristics of these pathways have already been mentioned in the previous section on target cell killing, since they are used by the effector CD8 T cells to kill infected cells and to re-establish homeostasis in mice and humans, as previously described. In this section, we will come back on some particular aspects that have not been discussed.

1. Activation-induced cell death

T cells can die through a process of activation-induced cell death (AICD), involving the death molecule Fas. AICD is provoked by the *in vitro* TCR re-stimulation of already activated and expanded T cells in the absence of appropriate co-stimulation, and depends on the death receptor pathway (Fas, TNFR1, TRAILR). Upon TCR re-stimulation, FasL is expressed on the same cell that expresses Fas or on neighbouring CD8 T cells, allowing for caspase-dependent apoptosis. The involvement of Fas in the return to homeostasis has been confirmed by the study of mice and men with mutations

in this pathway, as already described (Watanabe-Fukunaga, Brannan et al. 1992; Takahashi, Tanaka et al. 1994; Rieux-Laucat, Fischer et al. 2003). However, the *in vivo* contribution of Fas in recovery after viral infection has been challenged by the demonstration that contraction may be independent on Fas or FasL in the context of LCMV or herpes simplex infection (Lohman, Razvi et al. 1996; Zimmermann, Rawiel et al. 1996; Pellegrini, Belz et al. 2003). Furthermore, the concept of AICD *in vivo* has even been questioned since complete viral clearance would prevent any kind of TCR re-stimulation (Strasser and Pellegrini 2004). Thus, Fas-dependent apoptotic pathway is likely a mediator of T cell contraction, but is certainly not the only one and might even be dispensable in some models, especially in the context of acute infections.

2. Death by neglect

The second major pathway of T cell death involves the disappearance of appropriate survival signals, leading to activated cell-autonomous death (ACAD) or death by neglect. Recently, this pathway has been shown to be a major player in T cell contraction. Upon pro-survival cytokine deprivation, the expression of the pro-apoptotic Bcl-2 family members Bim (Bcl-2 interacting mediator of cell death) and Puma (p53-upregulated modulator of apoptosis) is increased, leading to cell death by the intrinsic pathway (Hildeman, Zhu et al. 2002; You, Pellegrini et al. 2006). Bim and Puma bind to Bcl-2 or Bcl-X_L at the mitochondrial membrane, abrogating the inhibition of Bax and Bak, and thus promoting the release of cytochrome c (Willis, Fletcher et al. 2007). The balance of Bim/Puma versus Bcl-2/Bcl-X_L thus regulates T cell death. Interestingly, TCR stimulation increases Bim, suggesting that this pathway is also involved in AICD (Sandalova, Wei et al. 2004). The relevance of Bim in termination of the immune response after infectious challenge has been confirmed in multiple models (herpes simplex, MγHV, LCMV) (Pellegrini, Belz et al. 2003; Hughes, Belz et al. 2008; Weant, Michalek et al. 2008). Furthermore, recent papers suggest that the Fas pathway might complement the Bim pathway in chronic MγHV infection or acute LCMV infection (Hughes, Belz et al. 2008; Weant, Michalek et al. 2008). Interestingly, the role of these two complementary pathways in T cell homeostasis is demonstrated by the much more severe lymphoproliferative diseases that develop in mice when both genes are deleted.

3. Determinants of CD8 T cell contraction

In accordance with the prominent role of cytokine deprivation in T cell apoptosis, γ_c-dependent cytokines have been shown to regulate the extent of T cell contraction (Vella, Dow et al. 1998). In fact, γ_c-cytokine levels decrease as the antigen is cleared. Furthermore, proliferating T cells are more sensitive to cytokine withdrawal than resting T cells. Interestingly, recent *in vivo* studies have shown that treatment with IL-2, IL-7 and IL-15 delays the contraction phase and, in some cases, increases the number of memory cells generated (Blattman, Grayson et al. 2003; Yajima, Yoshihara et al. 2006; Nanjappa, Walent et al. 2008; Rubinstein, Lind et al. 2008). In fact, γ_c cytokines promote the expression of Bcl-2 and Bcl-X_L, counter-balancing Bim and Puma and preventing mitochondrial-induced cell death (Akbar, Borthwick et al. 1996).

CD8 T cell contraction appears also to be influenced by pro-inflammatory cytokines. In particular, IFNγ signals appear to be essential since IFNγ$^{-/-}$ or IFNGR1$^{-/-}$ mice fail to undergo contraction following LCMV or *Listeria* infection (Badovinac, Tvinnereim et al. 2000; Badovinac, Porter et al. 2004; Tewari, Nakayama et al. 2007). TNFα, another pro-inflammatory cytokine produced during viral infections, might also be involved in the regulation of CD8 T cell contraction (Suresh, Singh et al. 2005). Finally, as mentioned previously, perforin deficient mice develop severe lymphoproliferation upon viral challenge, exemplifying the role of this pathway in T cell homeostasis (Matloubian, Suresh et al. 1999; Badovinac, Hamilton et al. 2003). However, the dysregulated immune response in perforin$^{-/-}$ mice is probably related to an excess of expansion rather than an impaired contraction (Badovinac, Tvinnereim et al. 2000; Badovinac, Porter et al. 2002).

Two other aspects of CD8 T cell contraction are worth mentioning. First, it is interesting to note that the onset and magnitude of contraction is independent of the magnitude of the prior expansion and thus the dose or duration of the infection (Badovinac, Porter et al. 2002). It has been nicely demonstrated by Badovinac et al in an infectious model with attenuated strain of *Listeria monocytogenes*. After low or high dose of infection, and despite a ten-fold difference at the peak of the response, the onset and kinetic of contraction is exactly the same. Furthermore, treatment with antibiotics to decrease the antigen load or establishment of chronic infection does not perturb the timing and kinetic of the contraction phase (Badovinac, Porter et al. 2002). It is thus thought that CD8 T cell contraction might be programmed very early upon

antigen encounter, like its differentiation. Secondly, it has to be remembered that T cell contraction is not required for memory cell generation, since antibiotic treatments diminish significantly the strength of signal 1, abolish the expansion and thus the contraction phase, but not the generation of functional memory cells (Badovinac, Porter et al. 2004). The absence of inflammatory signals, especially IFNγ, might be involved in this process.

B. Cellular markers for the identification of precursor memory cells

One major unresolved question in CD8 T cell immunology is the understanding of the mechanisms involved in the lineage choices between the generation of potent effector responses and the selection of precursor cells destined to become long-lasting memory cells. Similarly, we still do not understand how apoptotic cell death is imposed on the majority of effector cells while preserving some of them from efficient elimination. The identification of memory precursor cells would enable us to study the initial events leading to the generation of stable memory. In fact, the advance in flow cytometry technologies has confirmed an important heterogeneity in the effector T cell pool. As we will see, numerous memory precursor identification markers have been proposed, but none unmistakably defines cells that will initiate the memory T cell pool.

1. IL7Rα

An important γ_c-dependent cytokine, IL-7, is essential for the survival and maintenance of both the naïve and memory CD8 T cell pool, through the regulation of the anti-apoptotic molecules Bcl-2 and Bcl-X_L (Schluns, Kieper et al. 2000; Goldrath, Sivakumar et al. 2002). As discussed previously, its receptor, composed of the IL7Rα (CD127) and γ_c chains, is downregulated soon after activation and is slowly reexpressed thereafter. In the quest for memory precursor cells, an important breakthrough came from two independent studies proposing that CD127 could be used as an identification marker (Kaech, Tan et al. 2003; Huster, Busch et al. 2004). In a LCMV infection model, Kaech et al. demonstrated that 5-10% of the effector T cell pool is expressing the CD127 chain at the peak of the response. Adoptive transfer studies of $CD127^{high}$ and $CD127^{low}$ cells confirmed that the $CD127^{high}$ cells were most effective in generating memory CD8 T cells in recipient mice. Subsequent studies demonstrated that the CD127 kinetic of upregulation was correlated to the strength of the signals received at

the time of activation (Badovinac, Porter et al. 2004; Lacombe, Hardy et al. 2005). Antibiotic treatment in the *Listeria* model or DC vaccination, two models leading to more rapid generation on memory cells, were also correlated with an increased frequency of $CD127^{high}$ effector cells at the peak of the response. Thus, the re-expression of CD127 was proposed to be an important identification marker of memory precursor cells.

However, some authors questioned the relevance of CD127 expression for memory generation. First, Badovinac et al. showed that the presence of CD127 on CD8 effector T cells did not prevent them to undergo T cell contraction (Badovinac, Messingham et al. 2005). Second, IL7 signals were found not to be essential for the selection of $CD127^{high}$ cells since $IL7^{-/-}$ effector CD8 T cells could upregulate CD127 (Klonowski, Williams et al. 2006). Lastly, in a very nice model of enforced expression of CD127 in a transgenic mouse, the presence of CD127 did not rescue the effector cells that were destined to die (Hand, Morre et al. 2007; Haring, Jing et al. 2008). Despite these reports, CD127 expression appears to be an important characteristic of memory T cells and might be useful, in conjunction with other markers, in the identification of memory precursor cells.

2. CD62L

One of the markers that could be associated with CD127 in the identification of memory precursors is CD62L (L-selectin). As discussed earlier, CD62L is important for the homing of CD8 T cells away from the infected tissues and in the peripheral lymphoid organs. In humans, expression of CD62L (in association with CCR7) was strongly correlated with a central memory phenotype, thus suggesting that CD62L might be able to identify memory precursor cells (Sallusto, Lenig et al. 1999). In fact, two studies confirmed that CD62L in association with CD127 could identify three effector populations with distinct characteristics ($CD62L^{low}CD127^{low}$, $CD62L^{low}CD127^{high}$, $CD62L^{high}CD127^{high}$) (Huster, Busch et al. 2004; Bachmann, Wolint et al. 2005). The $CD62L^{low}CD127^{low}$ effector cells are potent cytotoxic cells, while the $CD62L^{high}CD127^{high}$ effector cells have reduced cytotoxic functions but increased IL-2 secretion and proliferative potential after rechallenge, and thus resembles memory cells.

However, the relevance of CD62L expression for the identification of functional memory cells, and thus their precursors, was soon questioned. In fact, it appears that it is not the expression of CD62L that defines the functional memory cell pool but the time passed since infection (Roberts, Ely et al. 2005). Furthermore, the kinetic of CD62L expression is influenced by early events at the time of priming, such as antigen levels, clonal competition, duration of infection and associated inflammatory environment (Wherry, Teichgraber et al. 2003; Marzo, Klonowski et al. 2005; Sarkar, Teichgraber et al. 2007; Wirth, Pham et al. 2009). Finally, in secondary immune responses, CD62L can no longer be used as a marker of memory cells, since secondary CD8 effector cells present a significant delay in their CD62L re-expression capacity while providing potent protection against rechallenge (Jabbari and Harty 2006). Thus, the absence or presence of CD62L expression on memory cells might not always be correlated with potent protective functions. However, its re-expression might be an added tool in the search for memory precursors.

3. KLRG1

In recent years, low expression of KLRG1 on effector cells was proposed as a new marker for the identification of memory precursor cells (Joshi, Cui et al. 2007; Sarkar, Kalia et al. 2008). KLRG1, for killer cell lectin-like receptor G1, belongs to the C-type lectin-like superfamily and is expressed by NK cells and T cells. It was recently shown to bind cadherins, although the impact of such binding *in vivo* is still not quite understood (Ito, Maruyama et al. 2006; Rosshart, Hofmann et al. 2008). Its expression is dramatically increased after infections and is suggested to be a marker of terminal differentiation of both cell types (Robbins, Terrizzi et al. 2003). In fact, extensive numbers of cell divisions are required for KLRG1 to be expressed. Furthermore, T cells expressing KLRG1 are potent killers but are unable to proliferate upon antigen challenge, thus identifying potent but senescent cytotoxic cells (Voehringer, Blaser et al. 2001). In a very nice study, Ahmed's group suggested that KLRG1 expression could be used as an early marker of effector or memory precursor cells, at a time when CD127 is still undetectable (Sarkar, Kalia et al. 2008). They proposed that KLRG1^{high} CD127^{low} short-lived effector cells (SLECs) are destined to become terminal effector cells while KLRG1^{low} CD127^{high} memory precursor effector cells (MPECs) will become long-lived memory cells. Through transfer studies of KLRG1^{high} and KLRG1^{low} cells, they demonstrated that KLRG1^{low} cells had a greater potential to become memory cells, and secreted more IL-2 than their KLRG1^{high} counterparts. Interestingly, both subtypes were

potent effector cells, with similar granzyme B expression, IFNγ and TNFα secretion and thus direct *ex vivo* killing. Furthermore, curtailing the antigenic stimulation, by decreasing the exposure time to the pathogen, promoted the development of KLRG1^{low} CD127^{high} MPECs, in accordance with quicker memory cell generation in this context. Interestingly, other studies had already suggested that the inflammatory environment at the time of priming impacted on the development of SLECs versus MPECs through the regulation of T-bet expression (Joshi, Cui et al. 2007). By studying T-bet$^{-/-}$ mice, they confirmed that T-bet was necessary and sufficient for the development of SLECs (Joshi, Cui et al. 2007). They also demonstrated that IL-15 signals were essential for the development of SLECs, thus suggesting a complex interplay between γ_c-cytokines pathways, downstream transcription factors and T cell lineage choices.

Although these three markers are helpful tools, we have to remember that they do not unequivocally identify memory precursors cells. While, as we suggested, CD127 expression is not sufficient for memory cell generation, CD62L expression does not always equal with presence or absence of memory functions, especially in secondary immune responses. Furthermore, the KLRG1/CD127 dichotomy is not absolute, since all of the KLRG1^{low} CD127^{high} MPECs do not become memory cells and some memory cells still express high levels of KLRG1. Thus, care has to be taken while studying mechanisms involved in memory generation since the precise identification of memory precursor cells is still under intense scrutiny.

C. Inflammatory milieu and rate of memory development

Numerous recent studies have suggested that the inflammatory environment present at the time of priming not only influences the differentiation and the contraction phase, but impacts on the time required to develop functional memory cells. In fact, memory CD8 T cell development is a gradual process, an on-going differentiation path to attain potent protective functions. The rate at which CD8 T cells acquire these memory characteristics is highly influenced by pro-inflammatory cytokines. In the reports by Badovinac et al, presented earlier in the 'contraction' section, antibiotic pre-treatment in the *Listeria* model diminishes the inflammatory cytokine production and the duration of antigenic display, without influencing the number of memory cells generated. In fact, mice develop phenotypic and functional characteristics of memory cells within one week, thanks to the significant reduction in the inflammatory signals. More importantly, CD8 T cells in the antibiotic treated mice were capable of vigorous

expansion at seven days after the initial infection, while the control group could similarly expand much later, being at the peak of their effector response at that time, and thus much less responsive to booster immunization. Interestingly, CpG treatment to restore inflammation prevents the rapid development of memory cells and extends the time required to gain memory functions (Badovinac, Porter et al. 2004; Badovinac and Harty 2007). Similarly, peptide-loaded DCs immunization provides a setting where CD8 T cells receive potent signals in the absence of inflammation. Consistent with the idea that inflammation influences the rate of memory generation, CD8 T cells primed in this context differentiate rapidly to a memory phenotype and are able to proliferate vigorously in response to re-infection (Badovinac, Messingham et al. 2005). Interestingly, CD8 T cells that respond to LCMV infection in an $IL12^{-/-}$ or $IFN\gamma^{-/-}$ environment exhibit rapid acquisition of memory characteristics and functions (Badovinac, Porter et al. 2004; Badovinac, Messingham et al. 2005; Pearce and Shen 2007). Similarly, the inflammatory environment impacts on the expression of key transcription factors, such as T-bet. Low degrees of inflammation will decrease the levels of T-bet expression and promote the development of MPECs and memory CD8 T cells (Joshi, Cui et al. 2007).

D. Memory cells functions and dysfunctions

1. Protective memory cells

The memory cells generated after the contraction phase persist for life and convey heightened protection after re-challenge. The protective capacity of memory CD8 T cells is dependent on multiple factors. First, as a consequence of clonal expansion, the number of antigen-specific precursors increases by 1000-fold (from 100-200 naïve cells to $5X10^5$ GP_{33-41}-specific memory cells per spleen in the context of LCMV infection) (Blattman, Antia et al. 2002). Moreover, the selected clones have a higher TCR affinity, decreasing the subsequent threshold required for activation (Busch and Pamer 1999). Second, compared to effector cells, memory cells persist in a pre-activated state with low levels of the cell cycle inhibitor $P27^{kip}$ and high levels of the cyclin-dependent kinase 6 (CDK6), allowing for their rapid transition from the G0/G1 phase to the S phase of the cell cycle (Veiga-Fernandes and Rocha 2004). Thus, the augmented precursor frequency and the strong proliferative capacities of memory cells provide prompt protection. Third, compared to naïve cells, memory cells have an

increased capacity to acquire effector functions following changes in their gene expression profile. Chromatin remodelling and activation of different transcriptions factors after priming allows for constitutive expression of some effector molecules such as IFNγ, perforin and granzyme B. Thus, elevated levels of those transcripts will endow memory CD8 T cells with the capacity to release large quantities of effector proteins rapidly (Araki, Wang et al. 2009). Fourth, the localization of memory cells near infectious entry sites favours rapid and efficient responses. In fact, primary activation and differentiation generate changes in the expression of adhesion and chemokine receptors, as well as tissue-specific localization imprinting. It allows for homing of memory cells in either non-lymphoid tissues or secondary lymphoid organs (Weninger, Crowley et al. 2001). Finally, CD8 memory T cells possess self-renewal capacities provided by cytokine-dependent homeostatic proliferation mechanisms (Homann, Teyton et al. 2001; Surh and Sprent 2008). The multipotency and renewal capacities of memory cells are characteristics shared with other types of stem cells. It allows for the generation of a long-lasting pool of highly amenable and responsive cells.

Similar to effector cells, significant heterogeneity exists among the CD8 memory T cell pool. As alluded previously, CD62L and CCR7 expression on memory T cells was suggested to distinguish two functionally distinct human memory T cell pools (Sallusto, Lenig et al. 1999). In humans, the central memory T cells (T_{CM}) express lymph node homing receptors ($CD62L^{high}$ $CCR7^{high}$), are less lytic than their $CD62L^{low}$ $CCR7^{low}$ counterparts, but proliferate more and secrete more IL-2 upon restimulation. The effector memory population (T_{EM}) is undistinguishable from the effector cells in terms of CD62L and CCR7 stainings, resides mostly in non-lymphoid peripheral tissues, next to infectious entry points, and is constituted of potent killers and cytokine producers (IFNγ, TNFα). In mice, despite the fact that the importance of CD62L and CCR7 for homing to peripheral lymphoid organs was established, this sub-division amongst memory cells is not as definite. The existence of memory cells in distant tissues was reported by numerous studies, but investigations on the distinct roles of each of these two memory subsets are still awaited (Masopust, Vezys et al. 2001). This paradigm is even more questionable in CD8 immunology since most authors reported nearly equivalent cytototoxicity and cytokine production of CD8 T_{CM} and T_{EM}. Yet, the heightened proliferative capacity of T_{CM} to antigen or γ_c-dependent cytokines has been described in some but not all types of infection (Unsoeld, Krautwald et al. 2002; Wherry, Teichgraber et al. 2003; Roberts and Woodland 2004; Roberts, Ely et al. 2005). Nevertheless, as suggested earlier, the kinetic of upregulation of CD62L is influenced by multiple factors. Time itself changes the expression profile of the memory

T cell pool. Furthermore, memory cells are probably not committed to stay exclusively in one site and are most likely moving from one area to another, thus sensing multiple environments that will in turn influence their cellular profile and function. At last, as discussed for the identification of precursors cells, the absence of definite surface or molecular markers for memory cells renders functional comparative studies more difficult. Protective response against re-challenge is probably the best demonstration for the presence of memory cells.

It is interesting to note that secondary memory CD8 T cells generated in the context of prime-boost vaccination strategies for example are distinct from primary memory cells. Upon re-infection, these secondary CD8 T cells undergo protracted contraction and present substantial delays in the upregulation of CD62L, as already discussed. Furthermore, they appear to be less sensitive to homeostatic signals than primary memory cells. However, on a single-cell basis, secondary memory CD8 T cells are more protective against re-infection and exhibit sustained granzyme B expression and cytotoxicity (Unsoeld and Pircher 2005; Jabbari and Harty 2006; Masopust, Ha et al. 2006). Thus, protective functions are influenced by multiple parameters, and the stimulation history impacts on the effector functions of the memory T cell pool.

2. Lethargic or helpless memory cells

Besides the evidence presented earlier on the minimal role for CD4 help in effector CD8 T cell differentiation, numerous studies have reported a surprising role for CD4 T cells in the generation of long-lived, functional CD8 memory T cells. Even in situations associated with high inflammatory environment, such as LCMV or *Listeria* infections, it appears that lack of CD4 help generates dysfunctional (lethargic) CD8 memory cells. These helpless memory cells respond poorly to secondary challenge, are unable to re-expand and to secrete inflammatory cytokines. Furthermore, upon antigen re-stimulation, helpless memory cells synthesize and express important quantities of TRAIL at their surface, leading to their deletion by their own death-receptor apoptotic pathway (Janssen, Droin et al. 2005). The mechanisms through which CD4 help influences CD8 memory generation are still a source of intense debate. Some authors suggest that CD4 help is required at the beginning of the response, in order to imprint a complete differentiation program to CD8 T cells (Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003). Interestingly, Williams et al demonstrated that IL-2 signals given at the time of priming were essential for the

secondary expansion of CD8 memory T cells (Williams, Tyznik et al. 2006). It is not known if these IL-2 signals are uniquely derived from CD4 cells, but this cytokine certainly contributes to the programming of functional memory CD8 T cells. However, other authors propose that CD4 help is also required for the maintenance of a functional CD8 T cell memory pool, since CD8 memory numbers and functionality deteriorate over extended period of time in a $CD4^{-/-}$ environment, with or without the presence of TRAIL (Sun, Williams et al. 2004; Badovinac, Messingham et al. 2006). Help signals might also influence chromatin remodelling and transcription factors since unhelped CD8 memory cells present hypoacetylation at its histone sites and express relatively elevated levels of T-bet (Intlekofer, Takemoto et al. 2007; Northrop, Wells et al. 2008). Furthermore, T-bet deficiency restores the function of helpless memory T cells and promotes the acquisition of a central memory phenotype. It thus appears that signals received from the CD4 T cell at the time of priming might be essential for the development of robust CD8 memory responses.

3. Exhausted memory cells

Although acute infections generate potent and protective responses, multiple studies have revealed that chronic infections lead to ineffective or poor-quality CD8 responses (Fuller and Zajac 2003; Wherry, Blattman et al. 2003; Fuller, Khanolkar et al. 2004). When primed in the context of chronic antigenic stimulation, CD8 T cells loose their ability to perform cytotoxic functions. The CD8 memory T cells generated will become functionally exhausted and unable to provide sterilizing immunity. This exhaustion is characterized by a hierarchical loss in effector function, from inactivation of IL-2 production to a complete disappearance of all effector functions. Persisting infection can even lead to deletion of the CD8 T cell pool. Besides these severe dysfunctions, the antigen-driven proliferation of the CD8 T cells is severely impaired by chronic infection, thus preventing adequate expansion upon re-infection. Exhaustion is further characterized by the surface expression of specific inhibitory receptors, such as PD-1, 2B4 and LAG3 (Blackburn, Shin et al. 2009). The importance of PD-1, a TNF receptor family member, in the dysfunctional phenotype of memory cells of chronically infected mice was nicely confirmed by treatment with PD-1L antibodies, as already discussed. Blocking the interaction between PD-1 and its ligand leads to enhance viral control, increased cytotoxicity and cytokine production and ameliorated proliferation against viral antigen (Barber, Wherry et al. 2006; Ha, Mueller et al. 2008). In the context of chronic infection, the long-term maintenance of the memory CD8 T pool is

also severely perturbed. First, effector CD8 T cells become unable to revert to a population of long-lived memory T_{CM} cells. Second, maintenance through γ_c-dependent homeostatic mechanisms is hampered by the decreased expression of the γ_c-dependent cytokine receptors and the increased resistance to their signals. Therefore, memory CD8 T cells require persistent antigenic stimulation for their maintenance (Wherry, Barber et al. 2004; Shin, Blackburn et al. 2007).

The mechanisms underlying the acquisition of such exhausted phenotype are still unresolved, however recent studies have shed a new light on the field. The absence of CD4 help or co-stimulatory signals (CD28, CD40L or 4-1BB) was shown to aggravate the impact of chronic antigenic stimulation on memory generation (Fuller, Khanolkar et al. 2004; Fuse, Zhang et al. 2008). Interestingly, CD4-derived IL-21 was recently suggested to be essential for the control of chronic infections (Elsaesser, Sauer et al. 2009; Yi, Du et al. 2009). IL-$21^{-/-}$ or IL-$21R^{-/-}$ mice present considerable and rapid functional exhaustion upon infection with LCMV clone 13 and are unable to clear the viral infection. Likewise, IL-21 treatment of chronically infected $CD4^{-/-}$ mice rescues the functionality of the CD8 T cell pool and increases viral clearance. Besides the role of IL-21, the heightened inflammatory milieu present in the context of chronic infection might also negatively influence the differentiation of the CD8 memory T cell pool, since inflammation hinders the acquisition of memory functions. Finally, a recent study demonstrated that Blimp-1, a transcriptional repressor, is overexpressed in virus-specific CD8 T cells in the context of chronic infections (Shin, Blackburn et al. 2009). High levels of Blimp-1 are correlated with increased expression of inhibitory receptors and decreased viral clearance. Thus, Blimp-1 appears to be one of the transcription factors involved in the regulation of exhausted CD8 T cells.

E. Transcription factors and lineage choice

Identification of key proteins and transcription factors involved in the transition from an effector to a memory cell has been the focus of major attention. Studies evaluating naïve, effector and memory cell populations have revealed major changes in the gene expression profile through differentiation. Thus, CD8 memory cells have a unique gene-expression profile and, as we discussed, unique functions (Kaech, Hemby et al. 2002). Several transcription factors have been shown to influence the differentiation of CD8 T cells, and its transition from effector to memory cell. In fact, some transcription factors influence the lineage choice of a cell and promote the

development of either terminally differentiated SLECs and T_{EM} or less differentiated, long-lived MPECs and T_{CM}.

T-bet and its sister eomesodermin are the prototypic transcription factors involved in these cell-fate decisions. The T-box transcription factor T-bet, encoded by *tbx21*, was initially described for its role in Th1 differentiation. Conversely, eomesodermin, its sister encoded by *eomes*, is expressed specifically by activated CD8 T cells, but not by CD4 T cells. Both of them regulate the expression of genes encoding perforin, granzyme B and IFNγ, and thus are involved in CD8-mediated cytotoxicity and viral clearance (Pearce, Mullen et al. 2003; Sullivan, Juedes et al. 2003). Interestingly, CD8 effector differentiation was recently shown to occur in two distinct phases *in vitro*, with an early induction of T-bet and a late induction of eomesodermin, thus complementing each other (Cruz-Guilloty, Pipkin et al. 2009). Besides their role in the acquisition of potent effector functions, T-bet and eomesodermin are essential for the expression of the IL2Rβ chain on memory CD8 T cells and thus their response to IL-15 (Intlekofer, Takemoto et al. 2005). Hence, compound deficiency in both of these transcription factors leads to near complete loss of memory CD8 T cells. Recent studies have also revealed that T-bet promotes the development of SLECs over MPECs (Joshi, Cui et al. 2007). In fact, inflammatory signals induce the expression of T-bet, which in turn enhance KLRG1^{high} CD127^{low} SLECs. Interestingly, KLRG1 expression in NK cells is dependent on T-bet (Robbins, Tessmer et al. 2005). As expected, T-bet$^{-/-}$ mice are incapable of generating a KLRG1^{high} CD127^{low} SLEC population. T-bet also represses the expression of CD127 on CD8 T cells, thus diverting the differentiation further away from T_{CM} generation (Intlekofer, Takemoto et al. 2007). In fact, high levels of T-bet are present in both SLECs and T_{EM} cells, while eomes levels do not vary between subtypes of effector and memory cells, but increase with time (Intlekofer, Takemoto et al. 2007). Thus, T-bet deficiency, while preventing the development of T_{EM}, promotes the generation of potent and protective T_{CM}.

The regulation of these two transcription factors is complex and influenced by multiple inflammatory (IL-12, IFNγ) and γ_c-dependent (IL-4, IL-15, IL-21) cytokines, as well as TCR and co-stimulatory signals. Thus, priming and stimulatory signals might influence the outcome of a cell through the regulation of these two transcription factors. However, the impact of those cytokines is complex since some of them might have divergent actions. Hence, IL-12 promotes the expression of T-bet but downregulates the expression of eomesodermin (Takemoto, Intlekofer et al. 2006). Likewise, IL-21 represses the expression of eomesodermin while inducing the

expression of T-bet (Strengell, Sareneva et al. 2002; Suto, Wurster et al. 2006). Thus, understanding the delicate balance involved in the regulation of these transcription factors will enable us to influence the generation of potent effector cells and long-lasting immunity.

A second group of transcription factors involved in the differentiation and cell-fate decisions of CD8 T cells is Blimp-1, and its target Bcl-6. Blimp-1 was initially described for its crucial role in the differentiation and maintenance of B cells in mature plasma cells. Encoded by the gene *prdm1*, it represses transcription through modification of the chromatin structure. Besides its role in CD8 T cell homeostasis, Blimp-1 is a strong repressor of *IL-2* gene transcription (Kallies, Hawkins et al. 2006; Martins, Cimmino et al. 2006). Actually, Blimp-1 itself is induced by IL-2 during the initiation of the immune response and appears to be essential for the contraction phase. Like T-bet, Blimp-1 is expressed in KLRG1^{high} CD127^{low} SLECs and T_{EM} (Intlekofer, Takemoto et al. 2007; Rutishauser, Martins et al. 2009). Interestingly, three recent studies have demonstrated that Blimp-1 is crucial for the lineage choice towards terminally differentiated SLECs and their ensuing effector memory cells (Kallies, Xin et al. 2009; Rutishauser, Martins et al. 2009; Shin, Blackburn et al. 2009). In fact, CD8 T cells from Blimp-1 deficient mice infected with LCMV or influenza virus fail to differentiate into SLECs. They display a defect in the granule exocytosis target-cell killing pathway, through downregulation of perforin and granzyme B, thus suggesting that Blimp-1 is crucial for the acquisition of cytotoxic functions. Moreover, Blimp-1$^{-/-}$ CD8 T cells present a migration defect with increased expression of CCR7 and decreased expression of CCR5 and thus inability to home to peripherally infected tissues. As for T-bet, Blimp-1 differentiates CD8 T cells towards effector cell and away from memory cell generation. Interestingly, its suppression promotes the expression of eomesodermin and Bcl-6, which are both associated with memory cell development. However, Blimp-1 is essential for the secondary expansion of memory CD8 T cells following viral challenge. In fact, although Blimp-1$^{-/-}$ cells are present in higher numbers and exhibit a central memory phenotype, they are unable to expand upon rechallenge. Thus, Blimp-1 shares some properties with T-bet for the differentiation of SLECs but has a distinct role in the generation of secondary immune responses.

Conversely, Bcl-6, another transcription repressor essential for the formation of memory B cells, is essential for the generation and maintenance of CD8 memory cells, especially T_{CM}. Transgenic expression of Bcl-6 increases the number of memory cells and T_{CM} upon viral infection, while Bcl-6 deficiency decreases their number (Ichii,

Sakamoto et al. 2004). Furthermore, Bcl-6 promotes the secondary expansion of the T_{CM} population upon rechallenge. Since Bcl-6 was recently shown to downregulate the expression of granzyme B, and is suppressed by Blimp-1, it is likely to be involved in the lineage choices of a CD8 T cell, away from effector differentiation and towards memory generation (Yoshida, Sakamoto et al. 2006).

F. Memory T cell maintenance and γ_c-dependent cytokines

The stable maintenance of the memory CD8 T cell pool depends on the longevity and the intermittent turnover of the cells surviving the contraction phase. Although the survival of memory cells was once thought to require contact with the priming antigen, it is now clear that both the survival and basal proliferation of antigen-experienced memory CD8 T cells is MHC, antigen and TCR-independent (Lau, Jamieson et al. 1994; Mullbacher 1994; Tanchot, Lemonnier et al. 1997; Murali-Krishna, Lau et al. 1999; Leignadier, Hardy et al. 2008). In accordance with the elevated expression of CD122 and CD127 on a majority of memory CD8 T cells, it was demonstrated that, in fact, memory CD8 T cell homeostasis in lymphoreplete environment relies on two γ_c-dependent cytokines, IL-15 and IL-7.

IL-15 is essential for the slow basal turnover of memory CD8 T cells. IL-$15^{-/-}$ and IL15R$\alpha^{-/-}$ mice lack the naturally occurring CD122^{high} memory cells generated upon encounter with self-antigens (Lodolce, Boone et al. 1998; Kennedy, Glaccum et al. 2000). Transfer of those cells in an IL-15-deficient environment prevents basal proliferation and leads to rapid death of the transferred cells (Goldrath, Sivakumar et al. 2002; Judge, Zhang et al. 2002). Furthermore, IL-15 transgenic mice are found to have elevated numbers of CD122^{high} CD8 memory T cells (Fehniger, Suzuki et al. 2001). This dependency on IL-15 for basal proliferation was further confirmed for real antigen-specific memory CD8 T cells. Memory CD8 T cells generated after infection with LCMV, VSV or vaccinia virus in IL-$15^{-/-}$ mice fail to homeostatically proliferate and thus disappear gradually (Becker, Wherry et al. 2002; Goldrath, Sivakumar et al. 2002; Schluns, Williams et al. 2002). Furthermore, transfer of wild-type antigen-specific memory cells in an IL-15-deficient environment precludes basal proliferation and longevity of the cells. Besides its significant role in the intermittent turnover of the memory CD8 T cell pool, IL-15 may also support their survival by upregulating the anti-apoptotic molecule Bcl-2 (Wu, Lee et al. 2002; Berard, Brandt et al. 2003).

IL-7 is a key player in the homeostasis of CD8 memory T cells by supporting their survival, through upregulation of Bcl-2, as it does for naïve CD8 T cells (Schluns, Kieper et al. 2000). Its action on basal turnover is minimal since increased transgenic overexpression of IL-7, even in the context of IL-15 deficiency, increases significantly the number of $CD122^{high}$ memory cells but not their basal proliferation, as determined by Brdu labelling (Kieper, Tan et al. 2002). The crucial role for IL-7 was recently confirmed by the analysis of IL7R mutant mice, in which the signal transduction through the receptor and Stat5 was abolished (Carrio, Rolle et al. 2007; Osborne, Dhanji et al. 2007). Those mice are able to generate CD8 memory T cells upon infection; however, even in presence of IL-15, the memory cells disappear slowly. Overexpression of Bcl-2 rescues the slow death of these memory cells, thus confirming that IL-7 is essential for the survival of CD8 memory T cells (Carrio, Rolle et al. 2007). Interestingly, the IL-7-dependency for long-term survival is more pronounced for cells primed by infectious agents than by a combination of antigen plus adjuvant (Burkett, Koka et al. 2003). In fact, memory cells generated upon infection can survive for extended periods in the absence of IL-15 by relying on IL-7. Nonetheless, these memory cells will ultimately disappear when deprived of IL-15-dependent homeostatic proliferation (Becker, Wherry et al. 2002; Goldrath, Sivakumar et al. 2002; Schluns, Williams et al. 2002). Thus, in the context of antigen-driven memory homeostasis, memory CD8 T cells primarily rely on IL-15 for their slow turnover and on IL-7 for their long-term survival.

The role for IL-2 in CD8 memory T cell homeostasis is minimal. In fact, it is believed that the availability of IL-2 is probably too low to be physiologically relevant for the long-term maintenance of memory T cells. However, by ligation to the IL2Rβγ complex, IL-2 has been shown to compensate for the absence of IL-15 in $IL15^{-/-}$ mice, when given in supra-physiologic quantities. In fact, anti-IL-2 antibodies enhance the biological action of IL-2 and promote the basal proliferation of the $CD122^{high}$ memory CD8 population (Kamimura, Ueda et al. 2004; Boyman, Kovar et al. 2006). However, a clear role for IL-2 in the maintenance of antigen-dependent memory CD8 T cells remains to be proven.

Homeostatic mechanisms must be tightly regulated since maintenance of homeostasis requires to accommodate for the survival of the memory T cell pool and their slow turnover. In fact, it appears that the overall size of the memory T cell pool remains relatively constant over extended periods of time (Freitas and Rocha 2000). Thus, apoptotic mechanisms must be involved to regulate the size of the memory T cell

pool. As stated before, survival of the memory CD8 T cells depends primarily on high levels of Bcl-2, in response to γ_c-dependent signals. Bcl-X_L and Mcl-1 transcripts are also highly elevated in CD8 memory T cells. Apoptosis of some of the memory cells relies on the pro-apoptotic molecule Bim, as exemplified by the restoration of peripheral T cell homeostasis after infection in IL-7-deficient environment in the absence of Bim (Pellegrini, Bouillet et al. 2004). The balance between these two molecules will thus be determinant to keep constant the size of the CD8 memory T cell pool (Wojciechowski, Jordan et al. 2006; Wojciechowski, Tripathi et al. 2007). The stability in the total number of memory cells suggests also that influx of newly generated memory T cells would be accommodated by the reciprocal death of pre-existing ones (Selin, Lin et al. 1999). However, recent data challenged this notion and proposed that in fact the memory T cell pool increases after each encounter, principally by the augmentation of T_{EM} cells and their localisation in non-lymphoid tissues (Vezys, Yates et al. 2009). This would be more compatible with the idea that vaccinations to multiple pathogens would protect the host and would not harm him by reducing prior immunity. Although the mechanisms allowing for the accommodation of new incomers are not known, it is possible that time might change the sensitivity to γ_c-dependent cytokines, and thus might regulate the survival and turnover of the diverse memory T cell clones.

RESULTS

I. γ_c-dependent cytokines provide Bcl-2-dependent and Bcl-2-independent homeostatic signals to naive CD8 T cells

Complementary results to Article 1:

γ_c cytokines provide multiple homeostatic signals to naïve CD4 T cells. Masse GX*, Corcuff E*, Decaluwe H, Bommhardt U, Lantz O, Buer J, Di Santo JP. *Eur J Immunol 2007, 37: 1-11*. (*authors contributed equally)

Cytokines signalling through receptors sharing the γ_c chain, especially IL-7, are critical for the development and peripheral homeostasis of naïve T cells. Furthermore, IL-2, -7, -15 and -21 are pleiotropic factors that can play complimentary or overlapping roles in T cell homeostasis and immune responses to infection. However, identification of their precise function during an anti-viral immune response has been challenging. In fact, the study of mice defective in all γ_c-dependent pathways has been complicated by the development of severe autoimmunity secondary to the absence of regulatory T cells (Malek and Bayer 2004). Furthermore, the IL-7-dependent survival defect of $\gamma_c^{-/-}$ cells has hampered the understanding of its role during the differentiation of CD8 T cells. Its downstream effect on Bcl-2 is crucial for the development and homeostasis of both $\gamma_c^{-/-}$ and IL7R$\alpha^{-/-}$ T cells since Bcl-2 overexpression increases the peripheral T cell numbers in both mice (Akashi, Kondo et al. 1997; Kondo, Akashi et al. 1997; Maraskovsky, O'Reilly et al. 1997). Indeed, γ_c deficiency affects not only the survival of naïve T cells but also the function of regulatory T cells, and non transgenic $\gamma_c^{-/-}$ mice ultimately develop an autoimmune syndrome secondary to TCR reactivity to environmental antigens (Nakajima, Shores et al. 1997; Sharara, Andersson et al. 1997; Suzuki, Zhou et al. 1999). This autoimmunity severely limits the use of γ_c-deficient mice for assessing T cell immunity. In contrast, certain TCR transgenic mice on the recombination-activating gene (Rag) 2 deficient background harbor monoclonal populations of naïve $\gamma_c^{-/-}$ T cells that have little environmental cross-reactivity (Lantz, Grandjean et al. 2000).

This approach allowed us to demonstrate that $\gamma_c^{-/-}$ (and IL-7R$\alpha^{-/-}$) CD4 T cells failed to accumulate in peripheral lymphoid organs and that this defect was associated with enhanced apoptosis (**see Article 1 in Annexe**). Over-expression of the human Bcl-2 rescued the γ_c deficient T cells, by promoting IL-7-independent survival of the cells. However, the generated $\gamma_c^{-/-}$ naïve CD4 T cells remained small in size and had a

persistent decrease in the expression of MHC class I and ribosomal proteins. This homeostatic defect could not be corrected by the addition of activated protein kinase B, suspected to be involved in the metabolism and survival of naïve T cells. Despite these homeostatic defects, $\gamma_c^{-/-}$ CD4 T cells were able to expand and proliferate in response to specific antigens *in vitro*. Thus, these results suggest that γ_c cytokines provide Bcl-2-dependent and -independent signals to maintain the phenotype and homeostasis of the peripheral naïve CD4 T cell pool.

To follow-up on this study, we investigated the impact of γ_c deficiency on the biology of naïve CD8 T cells, bearing in mind that the generation of a $\gamma_c^{-/-}$ naïve CD8 T cell pool would provide us with means to study the roles for γ_c cytokines in anti-viral CD8 immune responses. Since the P14 mice model is the basis of this scientific work and is used throughout this thesis, it will be the only transgenic model presented here. However, the following results were also confirmed in another CD8 TCR transgenic mice model (Mata-Hari mice) and in a second CD4 model (OT2 mice).

P14 mice develop naïve CD8 αβ T cells specific for the envelope glycoprotein 33-41 (GP_{33-41}) of the lymphocytic choriomeningitis virus (LCMV) in the context of H-$2D^b$. In order to study the impact of γ_c deficiency on CD8 T cell homeostasis, we generated $Rag2^{-/-}$ $\gamma_c^{-/-}$ P14 mice (P14 $\gamma_c^{-/-}$). In the absence of γ_c, P14 CD8 SP T cells are hardly selected in the thymus and fail to accumulate in peripheral lymphoid organs (**Figure 15A-C**). These scarce peripheral $\gamma_c^{-/-}$ CD8 T cells are reduced by 85-fold but display a naive profile (data not shown). Moreover, they present a small size and reduced expression of MHC class I protein along with markedly reduced Bcl-2 levels, suggesting a survival defect (**Figure 15B**). We thus decided to rescue the naive γ_c-deficient CD8 T cell compartment, by enforcing the expression of the human Bcl-2 transgene in T cells. As described for CD4 T cells, Bcl-2 corrected the peripheral T cell defect in these mice, generating normal numbers of splenic monoclonal CD8 T cells. Yet, consistent with the inability of Bcl-2 to correct the DN2 block in the early development of $\gamma_c^{-/-}$ thymocytes, the number of total and CD8 SP thymocytes stayed below normal (by 6-fold and 3-fold respectively) (**Figure 15A-C**). Cell surface expression markers were not modified by Bcl-2 overexpression, and the peripheral P14 Bcl-2 $\gamma_c^{-/-}$ CD8 T cells conserved a naïve phenotype (**Figure 15D**). They however remained small in size and expressed slightly lower levels of MHC class I, confirming the requirements for γ_c-dependent but Bcl-2-independent signals in some aspects of CD8 T cell homeostasis.

Thus, it appears that CD4 and CD8 naïve T cells rely on the same cytokine-dependent signals for their peripheral homeostasis. Furthermore, Bcl-2 overexpression rescues both compartments but does not correct the defects in size and protein synthesis seen in the context of γ_c deficiency. Finally, this transgenic approach allows us to generate a consistent population of naïve monoclonal $\gamma_c^{-/-}$ CD8 T cells that can be used to dissect the importance of γ_c signals in anti-viral immunity.

II. γ_c deficiency prevents CD8 T cell memory formation despite potent effector T cell development and function

Article 2:

γ_c deficiency precludes CD8 T cell memory despite formation of potent T cell effectors. Decaluwe H, Taillardet M, Corcuff E, Munitic I, Law HKW, Rocha B, Rivière Y, Di Santo JP. *Submitted to Proceedings of the National Academy of Sciences*.

The P14 Bcl-2 $\gamma_c^{-/-}$ model generated in the previous section is thus the ideal tool to study the role of γ_c-dependent cytokines on CD8 T cells throughout its differentiation program. In fact, studies on the isolated contribution of some of these cytokines during an anti-viral immune response have suggested a potential role for γ_c cytokines in the differentiation of CD8 T cells, during acute and/or chronic infections. Moreover, effector $\gamma_c^{-/-}$ CD4 T cells are severely hampered in their capacity to reject skin grafts, thus indicating that γ_c signals might be indispensable to the generation of potent cytotoxic functions (Masse, Corcuff et al. 2007). Finally, IL-7 and IL-15 were both shown to be essential for the long-term survival and basal proliferation of the memory CD8 T cell pool, through Bcl-2-dependent and -independent signals. Despite these results, the exact contribution of the γ_c-dependent cytokines in the differentiation of CD8 T cells remains disputed. Indeed, these cytokines are highly redundant, which might conceal the fundamental role for γ_c cytokines in the generation of effector and memory CD8 T cells. Moreover, it is unclear at which step of the differentiation process these cytokines impact and what is their importance on the cell-fate decision towards terminal differentiation versus memory generation.

In this study, we attempted to dissect the contribution of γ_c-dependent cytokines at each phase of an anti-viral immune response (**see Article 2 in Annexe**). Furthermore, because we had artificially corrected the γ_c-dependent survival defect of $\gamma_c^{-/-}$ T cells with the anti-apoptotic molecule Bcl-2, we assumed that this approach would allow us to analyse the impact of γ_c signalling on the generation and function of memory CD8 T cells. We thus adoptively transferred P14 Bcl-2 γ_c-competent or γ_c-deficient CD8 T cells into naive wild type (WT) recipients and infected them with the Armstrong strain of LCMV (see **Figure 16** for experimental design). In contrast to what was expected, we

found that many aspects of the antigen-driven CD8 T cell primary immune response proceeded normally in the absence of γ_c, including the initial clonal expansion and the classical phenotypic changes associated with activation. Moreover, $\gamma_c^{-/-}$ CD8 T cells were potent killers, despite decreased levels of granzyme B. Nevertheless, γ_c-dependent signals were necessary for the transition from effector to memory cell, affecting the differentiation and late proliferation of KLRG1^{high} CD127^{low} short-lived effector cells (SLEC). Furthermore, despite the presence of Bcl-2^{+} KLRG1^{low} CD127^{high} long-lived memory precursors (MPEC), γ_c signals were essential for the generation of memory cells. Together, our results define the critical stages for γ_c cytokines in the programming of terminal effector CD8 T cells and in the Bcl-2-independent survival and homeostatic proliferation of memory CD8 T cells.

III. IL-2 and IL-15 are dispensable for primary and secondary CD8 T cell immune responses

Supplementary results:
IL2Rβ signals promote CD8 T cell memory formation but are dispensable for potent primary and secondary immune responses. *Ongoing experiments, manuscript in preparation*.

Since each γ_c-dependent cytokine has distinct and redundant roles in the differentiation of CD8 T cells, we were tempted to analyze the impact of IL2Rβ deficiency on the primary and secondary immune responses to viral infection. The IL2Rβ chain, like the γ_c chain, is shared by both IL-2 and IL-15 cytokines, and forms with it an intermediate affinity receptor. The high affinity receptor is heterotrimeric and gives its cytokine specificity by the ligation with its third subunit, being the IL2Rα chain (CD25) for IL-2 and the IL15Rα chain for IL-15. Despite these common cytokine receptor subunits and downstream signalling molecules, IL-2 and IL-15 have unique functions, the most important of them being the absolute requirement for IL-2 signals in the maintenance of regulatory T cells and the essential role for IL-15 signals in the basal proliferation of memory CD8 T cells. Even though the independent role for IL-2 and IL-15 appears to be minimal in the differentiation of CD8 T cells, it is likely that the combined contribution of these two cytokines influence the expansion and cytotoxic functions of effector CD8 T cells. Furthermore, in the CD4-mediated model of graft rejection, the combined effect of IL-2 and IL-15 deficiency was more pronounced than the isolated impact of each of them. Lastly, the presence of IL-7 signals in the IL2Rβ$^{-/-}$ mice, as opposed to the $\gamma_c^{-/-}$ mice, might allow us to dissect the role for these two γ_c cytokines in the function of memory CD8 T cells, which we were not able to do in the previous model.

Thus, we adoptively transferred P14 IL2Rβ-competent and deficient CD8 T cells in naïve WT mice and infected them with LCMV Armstrong. Although IL2Rβ$^{+/-}$ and IL2Rβ$^{-/-}$ cells proliferated initially with the same kinetics, the peak of expansion was significantly reduced in IL2Rβ$^{-/-}$ CD8 T cells (32 X 10^6 versus 9 X 10^6 antigen-specific cells respectively, $p<0.0001$) (**Fig 17A**). Interestingly, the cell surface expression of

numerous activation markers was similar in both subsets, although differences were noted in terms of KLRG1, CD27, CD62L, CD127 and granzyme B expression, as seen in P14 $\gamma_c^{-/-}$ cells (**Fig 17B** and data not shown). A preferential accumulation of KLRG1^{low} CD127^{high} MPECs over KLRG1^{high} CD127^{low} SLECs is seen in IL2Rβ-deficient mice and the granzyme B defect is once again restricted to the KLRG1^{low} cells (**Fig 17C**). Moreover, cytokine production was evaluated by intracellular cytokine staining and similar proportions of IL2Rβ$^{-/-}$ CD8 effector T cells produced IFNγ or TNFα following *in vitro* restimulation as their IL2Rβ$^{+/-}$ counterparts, although 2-fold more IL2Rβ$^{-/-}$ cells secreted IL-2 (**Fig 17D**). We next evaluated the *in vivo* killing capacity of IL2Rβ$^{-/-}$ CD8 T cells in perforin-deficient (Pfp$^{-/-}$) chimeric mice. Despite the granzyme B defect, IL2Rβ$^{-/-}$ CD8 T cells were as potent killers as their IL2Rβ$^{+/-}$ counterparts (**Fig 17E**). Finally, to refine the evaluation of the effector function, we assessed if IL2Rβ$^{-/-}$ P14 T cells could prevent LCMV-induced hemophagocytic lymphohistiocytosis syndrome in Pfp$^{-/-}$ hosts. Pfp$^{-/-}$ recipients receiving either IL2Rβ$^{+/-}$ or IL2Rβ$^{-/-}$ CD8 T cells survived equally well through the period following LCMV infection and remained healthy, without developing any signs of the disease (**Fig 17F** and data not shown). Together, our data indicate that IL2Rβ signals condition the expansion and differentiation of KLRG1^{high} CD127^{low} SLEC but are dispensable for adequate effector functions, despite reduction in the granzyme B levels.

In order to evaluate if the abnormal differentiation of IL2Rβ$^{-/-}$ CD8 T cells had an impact on the development of memory T cells, we studied LCMV-infected chimeric mice more than 90 days after infection. This time, IL2Rβ$^{-/-}$ CD8 T cells were maintained after the contraction phase, for more than five months post infection. However, they were present in significantly lower proportions than their IL2Rβ$^{+/-}$ counterparts, with 0.5% of the blood CD8 T cells being IL2Rβ$^{-/-}$ compared to 6.5% IL2Rβ$^{+/-}$ cells ($p<0.0001$) (**Fig 18A**). At day 90 post infection, the spleen and bone marrow were similarly diminished in the numbers of memory IL2Rβ$^{-/-}$ CD8 T cells (**Fig 18B**).

To evaluate the functional capacity of these memory T cells, we reinfected the mice with the same dose of LCMV Armstrong. To our surprise, the P14 IL2Rβ$^{-/-}$ memory CD8 T cells were able to expand after reinfection, even though their proliferation was slightly diminished (**Fig 19A**). Interestingly, all of the IL2Rβ$^{-/-}$ memory CD8 T cells were KLRG1^{low} CD127^{high} CD62L^{high} prior to infection and few of them were able to increase their KLRG1 expression upon reinfection (**Fig 19B**). Functionally, the majority of secondary CD8 effector T cells were potent producers of IFNγ and TNFα, with or

without the IL2Rβ chain, with two-fold more IL2Rβ$^{-/-}$ cells being triple producers (IFNγ^{+} TNFα^{+} IL-2^{+} cells; p=0.02) (Fig 18C-E). Together, these results reveal an essential role for IL-2 and IL-15 cytokines in the generation of sufficient numbers of CD8 memory T cells, but appear to be in part dispensable for the expansion and acquisition of secondary effector characteristics. Future experiments will confirm if cytotoxic functions are preserved in IL2Rβ$^{-/-}$ memory CD8 T cells.

IV. Epitope specificity and relative clonal abundance do not affect CD8 T cell differentiation patterns during LCMV infection

Article 3:

Epitope specificity and relative clonal abundance do not affect CD8 T cell differentiation patterns during lymphocytic choriomeningitis virus infection. Munitic I*, Decaluwe H*, Evaristo C, Lemos S, Wlodarczyk M, Worth A, Le Bon A, Slin LK, Rivière Y, Di Santo JP, Borrow P, Rocha B. *J Virol 2009, 83: 11795-11807*. (*authors contributed equally)

All of the experiments presented above were done by adoptive transfer of 10^5 TCR transgenic CD8 T cells (specific for a dominant epitope of LCMV) in naïve WT recipients, followed by infection. However, controversies exist regarding this approach, since recent reports have suggested that transfer of high number of cells would alter the differentiation process. In fact, the precursor frequency for the GP_{33-41} epitope is in the order of 100-200 cells per spleen in a naïve WT mouse, while the precursor frequency after adoptive transfer is considered to be around 10% of the number of transferred cells (thus 50-100-fold more than WT precursor frequency in our case) (Blattman, Antia et al. 2002). Marzo et al proposed that high precursor frequencies would favor the rapid acquisition of T_{CM} phenotype and that only low precursor frequency would mimic the endogenous response (Marzo, Klonowski et al. 2005). Badovinac et al further insisted on the fact that changes in precursor frequency will influence the kinetic of expansion and the cell surface expression of activation and cytotoxic markers, while not affecting the peak number of effector cells or the extent of contraction (Badovinac, Haring et al. 2007). Despite these findings, it remains unclear if differences attributed to high precursor frequencies reflect changes in the differentiation pathways, as suggested, or if they were only a consequence of altered kinetics of differentiation. Thus, we were interested in evaluating the differentiation pattern of endogenous or transgenic CD8 T cells upon LCMV infection. We also assessed the different endogenous populations stimulated by LCMV, since some clones respond to dominant epitopes, while other to sub-dominant epitopes, creating a hierarchy in the response.

In this study, we evaluated the differentiation profile of endogenous CD8 T cells specific for two dominant epitopes and one sub-dominant epitope of LCMV, along with TCR transgenic T cells transferred in low-dose or high-dose numbers, at effector and memory time points (**see Article 3 in Annexe**). To increase functional discrimination, we assessed the differentiation process with a sensitive single-cell multiplex RT-PCR method, allowing for the screening of all CD8 effector genes simultaneously in single cells (Peixoto, Monteiro et al. 2004). Surprisingly, endogenous CD8 T cells with different epitope specificities exhibited similar differentiation patterns at day 4.5, 8 and 60. Furthermore, when comparing the endogenous and transgenic response to the same epitope in the same mice, the expression profile of effector genes was similar. The presence of GP_{33-41} transgenic cells decreased the expansion of the GP_{33-41}-specific endogenous compartment, but did not alter the response to the other epitopes, nor the immunodominance hierarchy in the memory phase. Finally, we proposed that transfer of TCR transgenic cells with distinct congenic markers might be an essential tool since TCR downregulation is major at the early stages of the response and may hamper the detection of antigen-specific CD8 T cells. Altogether, our results demonstrate that distinct epitope specificity and precursor frequency do not affect the differentiation pattern of effector and memory CD8 T cells.

DISCUSSION

γ_c-dependent cytokines have multiple and diverse roles in immunity and are an essential component of the adaptive immune response to infections. While they are central to the development of mature T cells in the thymus, γ_c cytokines also influence T cell survival and homeostasis in the periphery (Ma, Koka et al. 2006; Surh and Sprent 2008; Takada and Jameson 2009). Furthermore, IL-2, IL-7, IL-15 and IL-21 modify the T cell differentiation process upon infection and potentiate the generation and maintenance of memory T cells (Schluns and Lefrancois 2003; Rochman, Spolski et al. 2009). A significant amount of literature has revealed overlapping roles for γ_c cytokines at different phases of an immune response. However, controversies remain on the absolute requirements for such cytokines during the expansion and differentiation stages of the response. The high level of redundancy in their functions might further eclipse their important role in T cell immunity. Moreover, distinct cytotoxic T cells (CD4 T_H1 cells, CD8 T cells) might differ in their response to γ_c cytokines, since the requirements for γ_c-dependent signals in CD4 and CD8 memory T cell homeostasis vary (Surh and Sprent 2008; Takada and Jameson 2009). In this last section, we will discuss the importance of γ_c signals for the homeostasis and immune response of CD8 T cells and highlight some particular situations in which the influence of these cytokines might be altered.

I. Naïve T cell homeostasis

In order to avoid the possible redundancy of γ_c signals in the homeostasis and immune response to infection, we chose to work with a model in which all γ_c signals would be abrogated. The $\gamma_c^{-/-}$ mouse is thus the best model, but the lack of regulatory T cells and subsequent autoimmunity hamper their use in experimental studies (Malek and Bayer 2004; Setoguchi, Hori et al. 2005). However, by crossing a $\gamma_c^{-/-}$ mouse with a $Rag2^{-/-}$ TCR transgenic mouse, such autoimmunity can be prevented (Lantz, Grandjean et al. 2000). Interestingly, our models of γ_c deficiency ($Rag2^{-/-}$ P14 $\gamma_c^{-/-}$ and $Rag2^{-/-}$ Ml $\gamma_c^{-/-}$ mice) confirm an essential and indispensable role for γ_c cytokines in T cell homeostasis. As such, we showed that γ_c cytokines are of central importance for the survival of naïve CD8 and CD4 T cells, through the maintenance of the anti-apoptotic molecule Bcl-2. Over-expression of Bcl-2 increases the number of mature $\gamma_c^{-/-}$ T cells to near normal numbers and allows for the generation of a stable and consistent naïve $\gamma_c^{-/-}$ T cell pool. This Bcl-2 rescue is consistent with previous studies reporting that Bcl-2 overexpression partly restores the number of peripheral T cells in IL-7Rα and γ_c deficient mice respectively (Akashi, Kondo et al. 1997; Kondo, Akashi et al. 1997; Maraskovsky,

O'Reilly et al. 1997). Furthermore, as demonstrated by others (Schluns, Kieper et al. 2000; Tan, Dudl et al. 2001), IL-7 appears to be the principal soluble factor involved in this process. Indeed, $Rag2^{-/-}$ $IL2R\beta^{-/-}$ P14 or Ml mice possess the same number of peripheral naïve CD8 or CD4 T cells as their $IL2R\beta^{+/+}$ counterparts, while $Rag2^{-/-}$ $IL7R\alpha^{-/-}$ Ml mice present a significant reduction in the number of peripheral CD4 T cells. Even though the contribution of other γ_c-dependent cytokines, such as IL-4, IL-9 and IL-21, cannot be formerly excluded, their importance in peripheral T cell survival appears limited (Surh and Sprent 2008; Takada and Jameson 2009).

Interestingly, our studies reveal a distinct need for γ_c-dependent signals in the peripheral homeostasis of naïve CD4 and CD8 T cells. In fact, the homeostatic requirements for γ_c cytokines appear to be more pronounced for naïve Ml CD4 T cells than they are for naïve P14 CD8 T cells. Without the γ_c chain, Ml CD4 cells are reduced by 100-fold in frequency and 250-fold in absolute numbers, while P14 CD8 T cells are reduced by 65-fold in frequency and 85-fold in absolute numbers. Furthermore, the restoration of the naïve $\gamma_c^{-/-}$ T cell pool upon Bcl-2 overexpression is partial in the Ml CD4 $\gamma_c^{-/-}$ model while it is complete in the P14 CD8 $\gamma_c^{-/-}$ model. Thus, CD4 T cells might be more sensitive to cytokine deprivation than CD8 T cells. This increased sensitivity to death could be secondary to the altered expression of anti- and pro-apoptotic molecules in the absence of γ_c signals thus tipping the balance away from survival and towards cell death (**Figure 3**). Interestingly, preliminary studies in the lab propose that Ml $\gamma_c^{-/-}$ and Ml Bcl-2 $\gamma_c^{-/-}$ splenocytes express higher levels of activated Bax compared to their $\gamma_c^{+/+}$ counterparts (Masse et al., unpublished). In contrast, transcriptional analysis of the Bcl-2-related and BH3-only molecules did not reveal a significant difference between P14 Bcl-2 $\gamma_c^{+/+}$ and $\gamma_c^{-/-}$ splenocytes (data not shown). The lack of such analysis in the Ml CD4 model prevents us to draw firm conclusions, but the differential expression of pro- and anti-apoptotic molecules in CD4 and CD8 $\gamma_c^{-/-}$ T cells might explain the increased dependency of the CD4 T cells for γ_c signals. In this respect, CD4 T cells might require other IL-7-dependent signals for their survival. The IL-7 dependent anti-apoptotic molecule Mcl-1 could be a potential candidate (Opferman, Letai et al. 2003).

Besides their importance in T cell survival, γ_c-dependent cytokines influence the homeostatic proliferation of the naïve T cell pool (Surh and Sprent 2008). The importance of homeostatic proliferation in these models stands from the fact that the TCR transgenic mice are generated on a Rag2-deficient background. Indeed, the absence of one T cell subtype increases the “sense of space” of the naïve transgenic

cells and the availability of growth factors (Freitas and Rocha 2000; Takada and Jameson 2009). Thus, the differences seen between the Ml CD4 $\gamma_c^{-/-}$ model and the P14 CD8 $\gamma_c^{-/-}$ model might also be attributed to a distinct sensitivity to γ_c cytokines for naïve CD4 and CD8 T cell homeostatic proliferation. Since both basal and homeostatic proliferation rely on Bcl-2-independent IL-7 signals (Schluns, Kieper et al. 2000; Tan, Dudl et al. 2001), it is possible that the incomplete rescue of the Ml CD4 T pool is related to its decreased capacity for homeostatic proliferation in the absence of γ_c signals. This is consistent with the notion that overexpression of Bcl-2 is not sufficient to compensate for the inability of naïve T cells to undergo lymphopenia-induced proliferation in IL-7 deficient hosts (Tan, Dudl et al. 2001; Osborne, Dhanji et al. 2007). Alternatively, the difference between the Ml CD4 and P14 CD8 model might be explained by the different TCR affinities of the cells, since homeostatic proliferation depends also on the intensity of TCR signalling (Ernst, Lee et al. 1999; Goldrath and Bevan 1999; Kieper, Burghardt et al. 2004; Hao, Legrand et al. 2006; Leitao, Freitas et al. 2009). It is thus possible that Ml CD4 T cells present an increased dependency for γ_c signals because of the weaker TCR affinity for their cognate peptide. This would be consistent with the observation that $\gamma_c^{-/-}$ OT2 CD4 T cells and $\gamma_c^{-/-}$ Mata-Hari CD8 T cells, which express very low affinity TCRs, are incompletely rescued by the Bcl-2 transgene (data not shown). Thus, our results suggest that the TCR affinity defines the degree of reliance on IL-7 signals by setting the threshold required for γ_c-dependent homeostatic proliferation.

II. Size and metabolism

γ_c cytokines are also central for regulating the size and metabolism of the naïve T cell pool (**Figure 3**). In fact, γ_c signals are trophic factors and are essential for the metabolism and protein synthesis of both CD4 and CD8 T cells (Barata, Silva et al. 2004; Cornish, Sinclair et al. 2006; Ostiguy, Allard et al. 2007). To maintain a resting and quiescent state, and sustain housekeeping functions, naïve T cells require a low but constant rate of energy metabolism (Fox, Hammerman et al. 2005). γ_c signals increase the capacity of the cell to take up extracellular nutrients to maintain its integrity. Interestingly, we showed that both CD4 and CD8 T cells are smaller in size, and express lower levels of MHC class I molecules at steady state, in the absence of γ_c signals. These are not cell-intrinsic defects since activation leads to potent cell growth (blasts) and increased protein synthesis. Moreover, overexpression of Bcl-2 does not

correct these anomalies, suggesting that Bcl-2-independent pathways are involved in the regulation of the size and metabolism of naïve T cells.

In fact, signal transduction from γ_c-containing receptors not only involves the Jak-Stat pathway, but also the phosphoinositide-3-kinase (PI3K), the protein kinase B (PKB, also know as AKT), the mitogen activated protein (MAP) kinase and the src family kinase pathways, resulting in additional transcription factors activation (**Figure 20**). The PI3K-PKB pathway is particularly important for preventing naïve T cell atrophy (Barata, Silva et al. 2004). Thus, in the Ml CD4 model, we attempted to correct the survival and size defect by transgenic expression of activated PKB. Surprisingly, the increase in cell number was minimal and the size difference was unaffected. Thus, even though the PKB pathway does regulate pro-survival and pro-growth signals, the impact of this pathway on the regulation of CD4 T cell homeostasis appears to be minimal. It is likely that other γ_c-dependent pathways regulate the size and metabolism of naïve T cells. The trophic effects of IL-7 signals, downstream of PI3K, involve the activation of mTOR (mammalian target of rapamycin) and the sustained expression of GLUT1 (a glucose transporter) (Rathmell, Farkash et al. 2001; Wofford, Wieman et al. 2008). Could other γ_c-dependent molecules, aside from PKB, regulate the activation and expression of those proteins? Indeed, the oncogenic kinases PIM1 and PIM2, which are downstream of Stat5, appear to be essential for protein translation, glycolysis and uptake of extracellular nutrients during naïve T cell homeostasis (Fox, Hammerman et al. 2005; Hammerman, Fox et al. 2005). Finally, since $\gamma_c^{-/-}$ T cells increase in size following activation, TCR and costimulatory signals most likely compensate for the lack of γ_c signals to allow for potent energy uptake during the expansion phase.

III. Expansion

Upon encounter with a pathogen, the naïve CD8 T cell embarks on a differentiation program leading to its activation, proliferation, differentiation, contraction and memory generation (**Figure 4**). Multiple parameters define the quality and extent of this immune response (Williams and Bevan 2007; Harty and Badovinac 2008). The *in vivo* role for γ_c cytokines in the initial activation and expansion phase has been debated. Interestingly, upon infection with the Armstrong stain of LCMV, γ_c-deficient P14 CD8 T cells proliferate and express the same levels of activation markers as their γ_c-competent counterparts, at least during the first five days. This confirms a surprising independence of all γ_c signals during the initial expansion phase of the

response. This initial autonomy from γ_c signals does not mean that such signals are not necessary for the fine tuning of the CD8 immune response. In fact, the presence of γ_c cytokine receptor subunits at the surface of the cell suggests a role for γ_c signals (**Figure 10**). However, our results are consistent with the notion that CD8 T cell expansion is programmed upon a short encounter with the pathogen (Kaech and Ahmed 2001; van Stipdonk, Lemmens et al. 2001; van Stipdonk, Hardenberg et al. 2003).

This *in vivo* independency from γ_c cytokines lasts 4-5 days, since clear differences appear thereafter. Thus, γ_c-deficient CD8 T cells proliferate less during the last days of the expansion phase, resulting in 10-times less cells at the peak of the response as compared to their γ_c-sufficient counterparts. This expansion anomaly is related to a diminished entry into cell cycle, but not to increased apoptosis. This is consistent with previous results showing that *in vitro* activated IL2Rβ-deficient cells express less $p27^{kip1}$ and cyclin D3 as compared to IL2Rβ-competent cells (Malek, Yu et al. 2001).

Which γ_c cytokines might be important for this late proliferative phase? IL-2 and IL-15 are likely the best candidates, since IL2R$\beta^{-/-}$ cells expand similarly to $\gamma_c^{-/-}$ cells. This is consistent with previous reports demonstrating that IL-2 signals might be essential to sustain the proliferation of CD8 T cells (D'Souza, Schluns et al. 2002; D'Souza and Lefrancois 2003; Verdeil, Puthier et al. 2006). In the IL2R$\alpha^{-/-}$ model reported by Williams et al, the absence of IL-2 signalling decreased the peak expansion of P14 cells by 2-fold (Williams, Tyznik et al. 2006). An IL-2R$\alpha^{-/-}$ chimeric mice model suggested a 5-fold difference between IL2Rα-deficient and competent cells (Bachmann, Wolint et al. 2007). Thus, our results imply that, in addition to IL-2 signals, IL-15 signals are likely necessary for potent expansion of the CD8 T cell pool, even though the requirements for IL-15 at this precise phase of the response have been controversial in the literature. In fact, dominant and sub-dominant antigen-specific clones respond differently to antigenic challenge in the absence of IL15Rα signals. Interestingly, GP_{33-41}-specific CD8 T cells rely on IL-15 signals for their complete expansion (Becker, Wherry et al. 2002). Finally, although there are no clear statistical differences between the peak number of P14 $\gamma_c^{-/-}$ and P14 IL2R$\beta^{-/-}$ effector T cells, the tendency for lower cell numbers at day 7 and 8 in the γ_c-deficient mice might suggest a role for IL-21 in the expansion of CD8 T cells, as proposed by others (Elsaesser, Sauer et al. 2009).

IV. Cytotoxic T cell differentiation

Even though γ_c signals appear to be dispensable during the initial phase of the response to LCMV, they might influence the programming and differentiation potential of the cells. In fact, numerous papers have suggested a role for γ_c cytokines in the acquisition of cytotoxic functions (Schluns and Lefrancois 2003; Glimcher, Townsend et al. 2004; Waldmann 2006; Spolski and Leonard 2008). In particular, IL-2 and more globally Stat-5 signals promote the perforin and granzyme B-dependent cytotoxicity of CD8 T cells primed by partial agonists (Verdeil, Puthier et al. 2006). Furthermore, peptide-based vaccination strategies reveal a significant contribution of IL-15 signals for the acquisition of CD8 cytotoxic functions (Rubinstein, Kadima et al. 2002). Finally, in the Ml model, γ_c-deficient CD4 T cells lack granzyme B and IFNγ expression and fail to reject skin grafts (Masse, Corcuff et al. 2007). Quite surprisingly, our results propose that γ_c cytokines (and in particular IL-2 and IL-15) are not essential for the acquisition of CD8 cytotoxic functions in the context of acute LCMV infection. γ_c and IL2Rβ-deficient CD8 T cells eliminate efficiently peptide-loaded target cells and prevent the development of lymphohistiocytosis syndrome in $Pfp^{-/-}$ mice, presumably through efficient elimination of the virus.

How could such potent responses be accounted for? Most likely, other co-stimulatory molecules present at the time of infection compensate for the lack of γ_c signals. Indeed, the role of γ_c cytokines in the acquisition of CD8 effector functions has been similarly demonstrated in the context of decreased "signal one" (weak agonists) or "signal three" (low inflammatory states)(Rubinstein, Kadima et al. 2002; Verdeil, Puthier et al. 2006). Since the combined signals received at the time of priming determine the extent of the differentiation potential (Haring, Badovinac et al. 2006; Williams and Bevan 2007), γ_c cytokines might be essential to complement the absence of other stimulatory signals. This theory would be coherent with the two following observations. First, numerous authors have demonstrated that CD4 help (involving CD40L and/or IL-2) might not be required for the primary immune response of CD8 T cells in the context of pathogenic infections, owing to the high pro-inflammatory signals associated with such infections (Buller, Holmes et al. 1987; Rahemtulla, Fung-Leung et al. 1991; Wu and Liu 1994; Le Bon, Etchart et al. 2003). Second, the apparent conflicts in the literature concerning the role of γ_c-cytokines (especially IL-2 and IL-15) might thus be attributed to the type of inflammatory signals generated by different pathogens, as demonstrated for IFNα signals (Thompson, Kolumam et al. 2006). It is likely that

infection with other viral or bacterial pathogens, such as vaccinia virus, herpes simplex virus or *Listeria monocytogenes*, lead to different results.

Interestingly, our results are also consistent with the notion that incomplete expansion does not preclude acquisition of effector functions. In fact, in a recent paper by Bevan's group, it was demonstrated that weak agonists promoted a curtailed proliferative response but were sufficient for proper differentiation of CD8 effectors (Zehn, Lee et al. 2009). Similarly, decreasing the duration of antigenic stimulation affected the magnitude of clonal expansion but not the functionality of the CD8 effector T cells (Prlic, Hernandez-Hoyos et al. 2006). Along these lines, γ_c-deprived antigenic stimulation would result in decreased overall strength of priming, leading to potent activation and effector differentiation, yet reduced clonal expansion.

An unexpected finding was the important difference between the Ml CD4 model and our P14 CD8 model in terms of effector T cell differentiation in the absence of γ_c. In fact, Ml $\gamma_c^{-/-}$ cells were unable to reject skin grafts and had profound anomalies in their differentiation profile (in terms of CD27, NKG2D, ICOS, IL12Rβ1 markers) and in the expression of IFNγ and cytotoxic molecules (Masse, Corcuff et al. 2007), while effector functions of P14 $\gamma_c^{-/-}$ cells were essentially normal. As stated above, the different context of activation, involving potent IFNα signals in the LCMV infectious model, might explain such differences (Kolumam, Thomas et al. 2005). Furthermore, CD4 T cells are distinctly different than CD8 T cells in their priming and activation requirements. Thus, CD4 T cells need longer antigenic stimulation and stronger co-stimulatory signals in order to embark on their differentiation pathway (Whitmire and Ahmed 2000; Foulds, Zenewicz et al. 2002). Moreover, the transcriptional signals required to become a potent T_H1 effector cell are more complex than the ones necessary for CD8 differentiation. These combined factors demonstrate that, as opposed to CD8 T cells, CD4 T cells most likely have a higher threshold for activation (Kaech, Wherry et al. 2002; Seder and Ahmed 2003). Thus, it seems possible that CD4 T cells need to integrate γ_c signals at the time of priming in order to complete their differentiation, while CD8 T cells have minimal requirements for them.

Despite these potent effector functions, $\gamma_c^{-/-}$ and IL2Rβ$^{-/-}$ CD8 T cells express lower levels of granzymes A and B transcripts (data not shown), and have less granzyme B granules per cell. These findings are consistent with previous reports demonstrating a role for IL-2 and IL-15 in the induction of granzyme B (Manyak, Norton et al. 1989; Ye, Young et al. 1996). Thus, it appears that granzyme B

transcription is influenced by γ_c cytokines. Which transcriptional molecules could be involved in this regulation, and how could γ_c cytokines influence these pathways are still unanswered questions. In fact, granzyme B gene transcription is influenced by numerous transcription factors (such as Ikaros, RUNX1, ETS, CREB1 and AP1) (Glimcher, Townsend et al. 2004). Recently, T-bet was shown to bind the granzyme B promoter in NK cells (Townsend, Weinmann et al. 2004), while eomes overexpression resulted in the induction of granzyme B expression in T_H2 cells (Pearce, Mullen et al. 2003). Furthermore, Blimp-1 deficient CD8 T cells failed to differentiate into potent effectors (Kallies, Xin et al. 2009; Rutishauser, Martins et al. 2009). Thus, these three important transcription factors might be targets of γ_c cytokines.

Although the precise signal-transduction mechanisms have not been thoroughly explored, γ_c-dependent regulation of granzyme B transcription likely involves the Stat5 pathway (Zhang, Scordi et al. 1999; Verdeil, Puthier et al. 2006). T-bet might also contribute to this regulation since its transcription is influenced by γ_c cytokines (our results and (Townsend, Weinmann et al. 2004; Suto, Wurster et al. 2006)). Finally, IL-2 was recently shown to induce Blimp-1 expression (Martins, Cimmino et al. 2008). Thus, Stat5, T-bet and Blimp-1 might be involved in the regulation of granzyme B transcription by γ_c cytokines. Given that these transcriptional pathways are also implicated in the regulation of perforin levels (Zhang, Scordi et al. 1999), we expect similar reduction in perforin expression in P14 $\gamma_c^{-/-}$ CD8 effector T cells. The reasons why this is not the case suggests that γ_c-independent signals might be sufficient for perforin synthesis.

V. Lineage choice

Throughout differentiation, CD8 T cells integrate signals to define their cellular fate (Kaech and Wherry 2007; Sarkar, Kalia et al. 2008). Since T-bet, eomesodermin and Blimp-1 are involved in the generation of potent effector functions, it is not surprising that these same transcription factors influence the lineage choice of a CD8 T cell. In fact, T-bet and Blimp-1 promote the generation of KLRG1^{high} CD127^{low} SLECs and its corresponding T_{EM} memory subtype (Intlekofer, Takemoto et al. 2007; Joshi, Cui et al. 2007; Rutishauser, Martins et al. 2009). Likewise, the absence of γ_c signals restricts the development of SLECs through the modulation of T-bet and possibly Blimp-1 (although this was not formerly tested). Furthermore, IL2R$\beta^{-/-}$ memory CD8 T cells present a strict T_{CM} phenotype (at least in terms of CD62L/CD127 expression). Thus, the

cytokines influencing the acquisition of an effector-specific lineage phenotype are most likely IL-2 and IL-15, which does not exclude the involvement of other γ_c-dependent cytokines, such as IL-21, might not be similarly involved. Interestingly, the levels of *Eomes* mRNA were not influenced by the absence of γ_c signals in P14 CD8 effector T cells. This is compatible with a recent report demonstrating that IL-2 signals increase *Eomes* levels in antigen-specific CD8 T cells stimulated *in vitro*, while IL-21 represses the IL-2-induced *Eomes* transcription (Hinrichs, Spolski et al. 2008). It is also consistent with the demonstration that *Eomes* levels are comparable in both SLECs and MPECs effector cells, and increase with time in memory T_{EM} and T_{CM} CD8 T cells (Intlekofer, Takemoto et al. 2007).

Even though the impact of γ_c signals in the terminal differentiation of effector T cells might be seen by some as trivial, we have to remember that the regulation of these important transcription factors is complex and influenced by multiple other inflammatory and co-stimulatory factors, which might complement the lack of γ_c signals. Furthermore, it would not be surprising to discover that all of these transcription factors are regulating one another, as suggested by others (Kallies, Xin et al. 2009). Analysing the transcriptional profile of $\gamma_c^{-/-}$ CD8 T cells activated through peptide-derived vaccinations could be a powerful approach to confirm our findings. Nevertheless, even in the context of acute LCMV infection, γ_c signals clearly promote the development of SLECs over MPECs at the peak of the response.

VI. Memory T cell homeostasis

During the contraction phase, a proportion of antigen-specific cells are selected to constitute the memory T cell pool. How some cells can escape the potent apoptotic mechanisms leading to disappearance of 90% of the effector T cell pool remains a mystery. In fact, the evidence demonstrating that the pro-apoptotic molecule Bim is one of the major actors of T cell contraction suggests that opposite anti-apoptotic molecules, such as Bcl-2 or Mcl-1 might be important for the survival of selected T cells (Hildeman, Zhu et al. 2002; Pellegrini, Belz et al. 2003). Since γ_c cytokines, and in particular IL-7, are important regulators of pro-survival genes transcription (Akbar, Borthwick et al. 1996), we could suspect that γ_c cytokines would influence the contraction phase and survival of the memory T cell pool. This seems to be the case since treatment with IL-2, IL-7 and IL-15 delays the contraction phase and in some cases increases the number of memory T cells (Blattman, Grayson et al. 2003; Yajima,

Yoshihara et al. 2006; Nanjappa, Walent et al. 2008; Rubinstein, Lind et al. 2008). Interestingly, the absence of γ_c signals and the concurrent presence of the Bcl-2 transgene during the contraction phase did not modify the slope of contraction in our model, but the timing and extent of contraction (data not shown). This is consistent with findings demonstrating that Bcl-2 overexpression does not prevent T cell contraction after a pathogenic challenge (Petschner, Zimmerman et al. 1998), suggesting that pro-apoptotic signals are strongly expressed and cannot easy be compensated for.

One hypothesis of our model was that the presence of the Bcl-2 transgene might be sufficient to rescue the $\gamma_c^{-/-}$ CD8 memory cells generated at the end of the contraction phase that would have otherwise died in the absence of IL-7-dependent pro-survival signals. Unexpectedly, despite the presence of Bcl-2, all of the γ_c deficient cells were eliminated by thirteen days post infection. The precocious loss of the γ_c deficient cells suggests that γ_c signals are mandatory for the generation of a memory T cell pool, through a Bcl-2 independent pathway. Interestingly, Osborne et al. recently showed that the presence of Bcl-2 in IL7Rα knock-in mice was not sufficient for maintenance of the memory T cell pool, although in these mice IL7Rα$^{-/-}$ memory cells could still be generated (Osborne, Dhanji et al. 2007). Furthermore, Bcl-2 did not appear to be strictly required for the maintenance of memory T cells in Bim$^{+/-}$ Bcl-2$^{-/-}$ mice, while its presence is necessary for cytokine-driven memory survival *in vitro* or memory T cell survival in lymphoreplete hosts (Wojciechowski, Tripathi et al. 2007). These surprising results suggest that other IL-7-dependent anti-apoptotic molecules might be essential for the survival of CD8 memory T cells and that the mere presence of Bcl-2 would not be sufficient to rescue $\gamma_c^{-/-}$ memory cells, in clear contrast with current thinking. Once again, the pro-apoptotic molecule Mcl-1 would be a good candidate, while Bcl-X_L is less likely to play a role in memory T cell homeostasis (Opferman, Letai et al. 2003; Zhang and He 2005).

Which γ_c cytokines could be involved in the regulation of memory CD8 T cell homeostasis? Undoubtedly, IL-7 is the principal candidate. In the IL2Rβ$^{-/-}$ model, the presence of memory CD8 T cells suggests a fundamental role for IL-7 in their generation and maintenance. However, the picture is probably much more complex, since deficiency in IL-2, IL-7, IL-15 or IL-21 leads to decreased number of CD8 memory T cells (Becker, Wherry et al. 2002; Schluns, Williams et al. 2002; Williams, Tyznik et al. 2006; Osborne, Dhanji et al. 2007; Elsaesser, Sauer et al. 2009; Yi, Du et al. 2009). Interestingly, suppression of both IL-15 and IL-7 signals only partially abrogates the

development of memory CD8 T cells (Kaech, Tan et al. 2003). The complete elimination of P14 $\gamma_c^{-/-}$ CD8 memory T cells upon viral infection suggests that a combination of these cytokines is likely involved.

The comparison between the percentage of remaining IL2Rβ$^{+/+}$ and IL2Rβ$^{-/-}$ cells at the end of the contraction phase is consistent with the fundamental role for IL-15 and/or IL-2 signals in the homeostasis of memory CD8 T cells. In fact, in the context of viral infections, the magnitude of the initial clonal burst typically determines memory T cell numbers (Badovinac, Porter et al. 2002). However, when correcting for the abrogated primary expansion of the IL2Rβ$^{-/-}$ T cell pool, a 10-fold difference persist in the number of memory CD8 T cells generated in the absence of the IL2Rβ chain (data not shown), suggesting that IL-15 (and/or IL-2) contribute to the maintenance of the memory T cell pool. In fact, IL-15 signals are fundamental for the homeostatic proliferation of CD8 memory T cells, and cannot be complemented by IL-7 (Becker, Wherry et al. 2002). Furthermore, IL-15 can also deliver pro-survival signals (Berard, Brandt et al. 2003). Despite the seemingly stable IL2Rβ$^{-/-}$ CD8 memory pool in the blood, as assessed by regular bleeding of the mice, longer follow-up or memory cell counts in other organs would likely demonstrate a slow decrease in the numbers of IL2Rβ$^{-/-}$ memory T cells with time. Furthermore, it would be interesting to evaluate if Bcl-2 over-expression in the IL2Rβ$^{-/-}$ model increases the number of memory T cells after LCMV infection, despite the absence of IL-15 signals.

VII. Recall responses

The memory cells generated provide heightened protection against reinfection. The role of γ_c cytokines in the modulation of these recall responses is still an area of intense investigation. Few studies have clearly looked at this particular aspect. One of the cornerstone studies on the subject reveals that IL-2 signals at the time of priming are essential for the survival and accumulation of CD8 memory cells upon reinfection, but not for their proliferation (Williams, Tyznik et al. 2006). Signalling through the intermediate affinity receptor IL2Rβγ during the primary immune response, independently of the IL2Rα chain, mimics this IL-2-dependent expansion. Interestingly, this is consistent with the requirements for CD4 help at the time of priming to achieve potent CD8 T cell recall responses at the memory phase (Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003). This is in clear contrast with the role of IL-15 signals in recall responses, since Becker et al. reported that IL15$^{-/-}$ CD8 memory cells are capable of potent expansion upon rechallenge (Becker, Wherry et al. 2002).

Knowing the fundamental role for IL-2 in the expansion of memory CD8 T cells upon recall, but the independence from IL-15 signals for such responses, we were surprised to see that IL2Rβ$^{-/-}$ memory CD8 T cells expanded as well as their IL2Rβ$^{+/+}$ counterparts upon reinfection. This is consistent with a previous report looking at the endogenous immune response to vaccinia virus in thymic-targeted transgenic IL2Rβ mice on an IL2Rβ$^{-/-}$ background (Yu JI 2003).

While IL-15 signals are dispensable for the expansion of memory CD8 T cells, they appear to influence the differentiation of those cells into potent secondary effector cells. In fact, IL-15 signals allow for quicker acquisition of killing functions through upregulation of granzyme B (Yajima, Nishimura et al. 2005). However, independently of IL-15, memory CD8 T cells upregulate granzyme B efficiently on the fifth day of re-infection. On the contrary, in the absence of CD4 help, memory CD8 T cells are incapable of cytokine secretion and cytotoxicity upon rechallenge (Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003). Once again, in clear contrast with these data, IL2Rβ$^{-/-}$ memory CD8 T cells not only expand after rechallenge, but secrete significant amounts of inflammatory cytokines and exhibit elevated protein levels of granzyme B. Confirmation of their potent cytotoxicity must be confirmed by proper testing of their specific killing abilities, but the presence of cytotoxic molecules in secondary IL2Rβ$^{-/-}$ cells suggest effective cytotoxic functions.

What could account for such differences? At this stage of the work, I can only speculate. Could it be that the memory CD8 population generated in the absence of IL-2 signals is different from the memory population generated in the absence of both IL-2 and IL-15 signals? A transcriptional analysis of the memory T cell pools in both contexts could help in answering this question. In fact, both T-bet and Blimp-1 influence memory T cell recall responses upon infection. While T-bet limits the expansion and protective responses of CD8 memory cells, Blimp-1 favors the acquisition of potent recall responses (Intlekofer, Takemoto et al. 2007; Kallies, Xin et al. 2009). Thus the relative expression of these two transcription factors could explain the distinct observations seen in the IL2Rα$^{-/-}$ and IL2Rβ$^{-/-}$ models. Future experiments will likely confirm this hypothesis.

CONCLUSION

The γ_c cytokine model of CD8 T cell differentiation

γ_c-dependent cytokines shape the CD8 immune response to LCMV infection and deliver essential signals for the proper programming and differentiation of CD8 T cells. These signals are distinct from co-stimulatory or pro-inflammatory signals and modulate specific phases of the response. As such, γ_c cytokines are essential for the late proliferation, the terminal differentiation of SLECs and the generation of long-lasting memory CD8 T cells. Depending of the infectious and inflammatory context, their influence might be minimized, but their activity is necessary to maximize the potential of the CD8 T cell pool. These γ_c-dependent signals also imprint specific cell fate decisions to the CD8 T cell pool, orienting the functional outcome of the anti-viral immune response.

At steady state, naïve CD8 T cells require Bcl-2-dependent IL-7 signals for their survival and Bcl-2-independent IL-7 signals for their growth, metabolism and protein synthesis. This second pathway might involve essential γ_c-dependent molecules of the PI3K pathway, aside from PKB, and of the Stat-5 pathway, possibly PM1/PM2, although these hypotheses have not been confirmed in the present work.

Upon encounter with a viral antigen, γ_c-dependent cytokines are dispensable for the initial proliferation and for the cytotoxic functions of CD8 T cells, leading to viral clearance. However, IL-2 and/or IL-15 are essential to maximize the proliferation of effector cells. Furthermore, these signals are important for the terminal differentiation of effector cells in granzyme B-expressing SLECs and for the subsequent generation of T_{EM} cells. To accomplish these functions, IL-2 and IL-15 probably act via different transcription factors, likely involving T-bet and Blimp-1.

Conversely, IL-7 signals promote the differentiation of MPECs and the subsequent generation and maintenance of T_{CM} cells. The survival function of IL-7 requires the expression of multiple anti-apoptotic molecules, since Bcl-2 expression is not sufficient for the survival of $\gamma_c^{-/-}$ CD8 memory T cells. Which transcription factors are modulated by IL-7 is unknown but eomesodermin and Bcl-6 might promote memory cell survival.

Other γ_c-dependent cytokines, in particular IL-21, are probably involved at different steps of these processes and might modify or potentiate the functions of other members of the same family of cytokines. Its indispensable role in chronic infections demonstrates the essential role for this cytokine in CD8 immune responses.

Overall, γ_c-dependent cytokines act in concert to orchestrate the most potent and effective T cell response and are involved in the generation of a long-lasting pool of fully functional memory CD8 T cells.

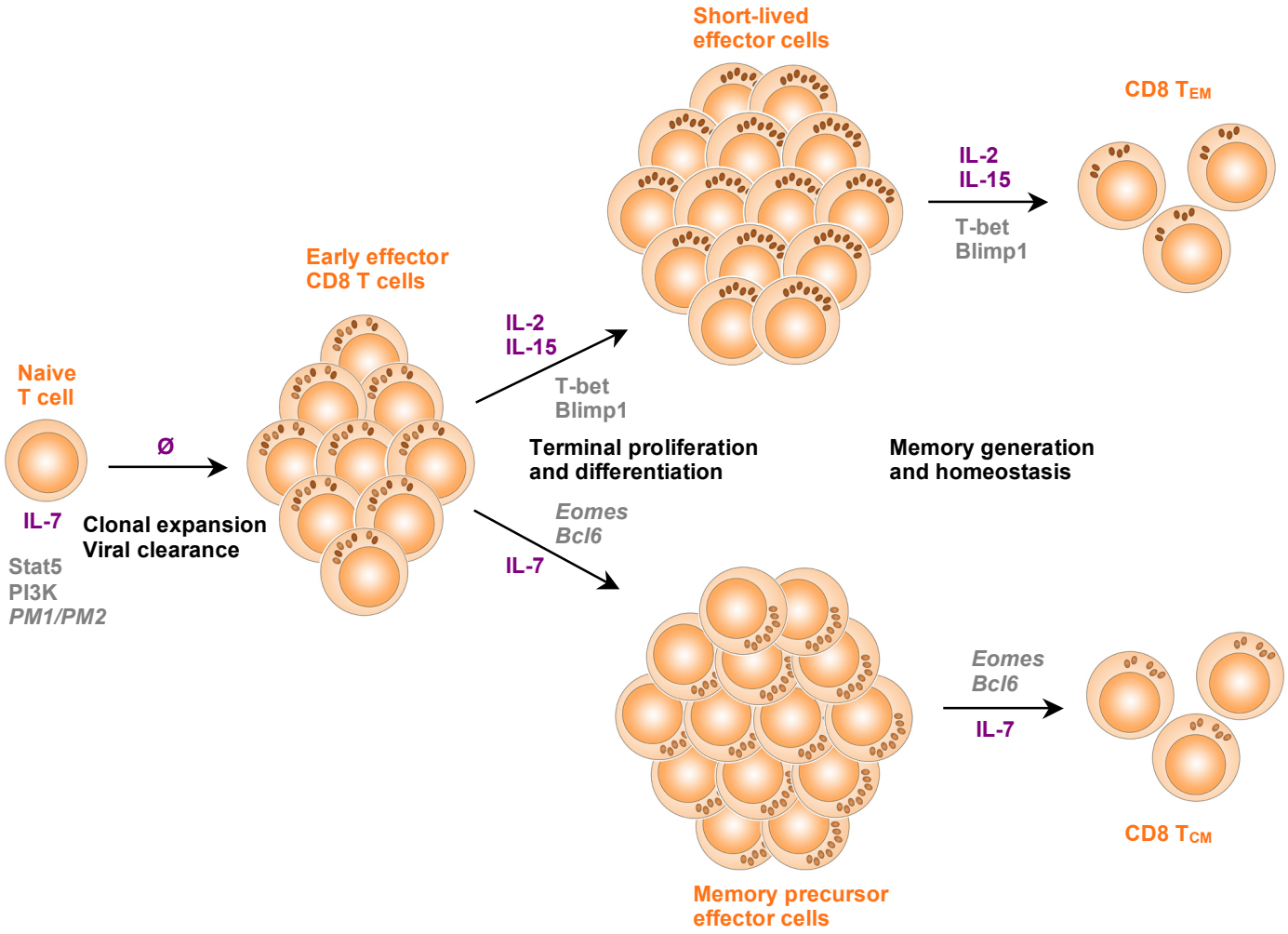

Figure 21: The γ_c cytokine model of CD8 T cell differentiation

γ_c-dependent cytokines shape the CD8 immune response to LCMV infection and deliver essential signals for the proper programming and differentiation of CD8 T cells. γ_c cytokines also regulate the growth and metabolism of naïve CD8 T cells and the generation and maintenance of a stable memory CD8 T cell pool.

From H. Decaluwe (unpublished)

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ANNEXE

I. Article 1: γ_c cytokines provide multiple homeostatic signals to naïve CD4 T cells

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γ_c cytokines provide multiple homeostatic signals to naive CD4$^+$ T cells

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Cytokines signaling through receptors sharing the common γ chain (γ_c), including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, are critical for the generation and peripheral homeostasis of B, T and NK cells. To identify unique or redundant roles for γ_c cytokines in naive CD4$^+$ T cells, we compared monoclonal populations of CD4$^+$ T cells from TCR-Tg mice that were γ_c^+, γ_c^-, CD127$^{-/-}$ or CD122$^{-/-}$. We found that γ_c^- naive CD4$^+$ T cells failed to accumulate in the peripheral lymphoid organs and the few remaining cells were characterized by small size, decreased expression of MHC class I and enhanced apoptosis. By over-expressing human Bcl-2, peripheral naive CD4$^+$ T cells that lack γ_c could be rescued. Bcl-2$^+$ γ_c^- CD4$^+$ T cells demonstrated enhanced survival characteristics *in vivo* and *in vitro*, and could proliferate normally *in vitro* in response to antigen. Nevertheless, Bcl-2$^+$ γ_c^- CD4$^+$ T cells remained small in size, and this phenotype was not corrected by enforced expression of an activated protein kinase B. We conclude that γ_c cytokines (primarily but not exclusively IL-7) provide Bcl-2-dependent as well as Bcl-2-independent signals to maintain the phenotype and homeostasis of the peripheral naive CD4$^+$ T cell pool.

Key words: Interleukin · Lymphocyte · Survival

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Introduction

Cytokines play essential roles in lymphocyte homeostasis. During lymphoid development, distinct cytokines have been identified that promote the survival and proliferation of B, T and NK cell precursors in the bone marrow and in the thymus. For example, IL-7 and stem cell factor are crucial for early thymocyte differentiation, IL-7 and fetal liver kinase-2 ligand drive B cell development, and IL-15 is essential for the generation of immature NK cells [1–5]. These observations indicate that cytokines have specific (*i.e.* non-redundant) roles during early lymphocyte development.

Cytokines that signal through the common γ chain (γ_c) include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Upon ligand binding, γ_c-containing receptors transmit intracellular signals through the JAK1 and JAK3 tyrosine kinases to activate primarily the transcription factors STAT3 and STAT5 [6]. Several γ_c-dependent receptors

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Abbreviations: **DN:** double-negative · **FSC:** forward scatter · **γc:** common γ chain · **Ml:** Marilyn TCR Tg mice · **PKB:** : protein kinase B · **SP:** single-positive

also signal through the PI3K, protein kinase B (PKB), MAPK and src family kinase pathways [7] and result in additional transcription factor activation. These different activation pathways converge in the nucleus where they modify gene expression profiles, although the impact of distinct transcription factors in this process is still unclear. Considering the complexity of γ_c signaling, it is not surprising that the transcriptional changes elicited by distinct γ_c cytokines include both unique and shared targets [8].

Downstream targets of γ_c signaling in lymphocytes include proteins involved in cell survival and apoptosis, such as members of the Bcl-2 family. This protein family includes both pro- and anti-apoptotic members; the latter inhibit the former by specific heterodimeric interactions [9]. Bcl-2 is a major target in early thymocyte development, especially during the IL-7-dependent 'double-negative (DN)2 stage [10]. In the absence of IL-7/γ_c, DN2 cells have decreased Bcl-2 levels, are prone to apoptosis and fail to proliferate normally [11].

Nevertheless, Bcl-2 is not the sole target of IL-7 signals at this stage, since over-expression of a Bcl-2 Tg in γ_c^- or CD127 (IL-7Rα)$^{-/-}$ mice does not correct the transitional DN2 block [12], although it improves the overall T cell homeostasis, especially in the periphery of IL-7 signaling-deficient animals [13, 14]. One possibility is that other Bcl-2 family members are also regulated by IL-7 in early thymocytes, including the pro-apoptotic Bax and/or Bad proteins [15]. Along these lines, mice made deficient in both CD127 and Bax demonstrated increased thymic cellularity compared with CD127$^-$ mice [16]. Finally, the anti-apoptotic protein Mcl-1 is also strongly up-regulated by IL-7 in thymocytes and peripheral T cells [17]. Mcl-1 acts independently of Bcl-2, and could explain the inability of enforced Bcl-2 expression to completely rescue the DN block in γ_c^- mice, or to allow recovery of other IL-7-dependent lymphocyte populations (*i.e.* $\gamma\delta$ T cells and B cells) in this context [18].

Once thymocytes complete the selection process and have fully differentiated as mature CD4 or CD8 single-positive (SP) thymocytes, they exit the thymus and re-circulate through the secondary lymphoid organs, including the lymph nodes and spleen. In the periphery, T cells require signals for their maintenance, which can be conveyed by TCR engagement, cell surface co-stimulatory receptors or soluble factors including cytokines. Of the latter, γ_c-dependent cytokines play critical roles for the homeostasis of naive $\alpha\beta$ T cells, $\gamma\delta$ T cells, NK and NKT cells. Adoptive transfer studies have shown that IL-7 supports peripheral survival and homeostatic proliferation of 'adaptive' CD4$^+$ and CD8$^+$ $\alpha\beta$ T cells, while IL-15 is essential for survival of 'innate' NK, NKT and $\gamma\delta$ T cells (reviewed in [19]).

The downstream effectors of IL-7 and IL-15 in this context remain to be fully defined, although these cytokines can regulate the expression of Bcl-2 family proteins [10, 20]. With respect to the role of IL-7 in naive T cell homeostasis, culture of human or mouse naive T cells *in vitro* with γ_c cytokines up-regulates Bcl-2 expression [21] and over-expression of Bcl-2 increases peripheral $\alpha\beta$ T cell numbers in CD127$^{-/-}$ mice [13, 14]. In contrast, Tg over-expression of Bcl-2 in γ_c^- T cells showed a limited effect on numbers of peripheral naive T cells [18]. This observation might indicate that γ_c-dependent cytokines other than IL-7 are involved in the homeostasis of naive T cells. Alternatively, these results might have been influenced by the propensity of γ_c-deficient mice to develop an inflammatory syndrome (secondary to generalized T cell activation resulting from an absence of regulatory T cells [22]). Our knowledge of the biological roles played by γ_c cytokines he maintenance of naive T cells remains incomplete.

In this report, we utilize Tg mice bearing the male antigen-specific Marilyn (Ml) TCR to assess the role for γ_c cytokines in the biology of naive CD4$^+$ T cells. In order to limit spurious TCR specificities resulting from associations of the Tg TCR β chain with endogenously rearranged TCR α polypeptides, all mice were RAG$^{-/-}$, and thereby harbored 'monoclonal' T cell populations. By comparing γ_c^+, γ_c^-, CD127$^{-/-}$ and CD122$^{-/-}$ Ml female mice, we define a series of γ_c-dependent phenotypes in peripheral naive CD4$^+$ T cells. By over-expressing Bcl-2 or a constitutively activated form of PKB in γ_c^+ and γ_c^- Ml CD4$^+$ T cells, we have further characterized the signaling pathways required for γ_c-dependent naive CD4$^+$ T cell homeostasis. Our results demonstrate that γ_c cytokines (primarily IL-7) provide Bcl-2-dependent as well as Bcl-2-indpendent signals to peripheral naive CD4$^+$ T cells.

Results

Phenotype of γ_c^- naive CD4$^+$ T cells

The TCR-Tg model system Ml on the RAG2$^{-/-}$ background generates monoclonal populations of CD4$^+$ $\alpha\beta$ T cells reactive with the male antigen Dby [23]. Peripheral CD4$^+$ T cells in Ml female Tg mice have a naive phenotype (CD44^{lo} CD62L^{hi}), are non-cycling and demonstrate limited homeostatic proliferation after transfer to lymphopenic recipients [24], consistent with their limited cross-reactivity to environmental and/or self antigens. In the absence of γ_c, CD4 SP Ml T cells are efficiently selected in the thymus, but fail to accumulate in the peripheral lymphoid organs [23]. These peripheral γ_c^- Ml CD4$^+$ T cells are reduced about 200-fold in comparison to their WT counterparts, although they demonstrate the expected naive CD44^{lo} CD62L^{hi}

phenotype. Moreover, γ_c^- Ml CD4$^+$ T cells in the spleen had markedly reduced Bcl-2 levels, suggesting a survival defect [23].

In order to gain more insights into the unique or redundant roles for γ_c cytokines in the homeostasis of naive CD4$^+$ T cells, we further characterized the cell surface phenotype of splenic CD4$^+$ Ml T cells that developed in the absence of γ_c (Fig. 1A–D). We found that splenic γ_c^+ and γ_c^- Ml CD4$^+$ T cells expressed similar levels of CD2, Vβ6, CD4, CD44 and CD62L and were negative for CD69 (Fig. 1A, B and data not shown).

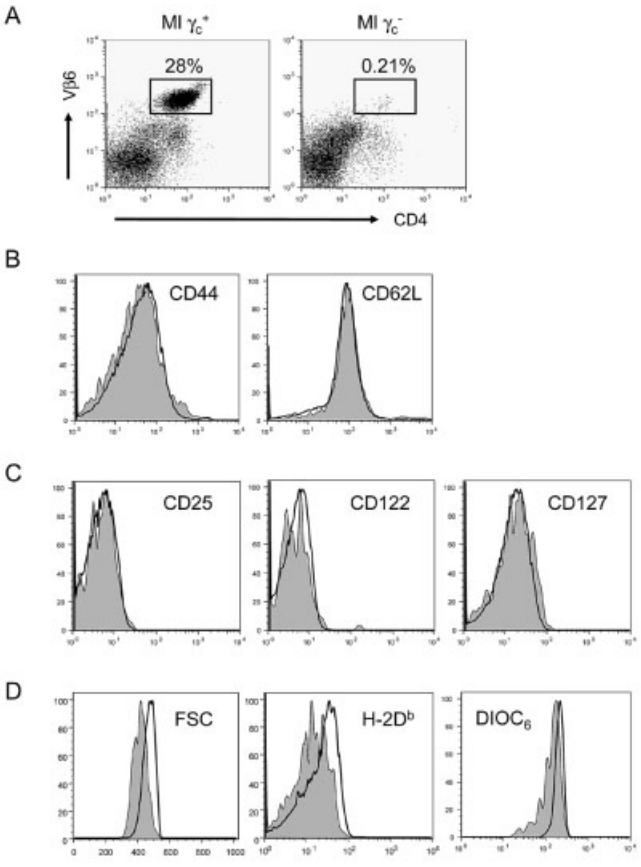

Figure 1. Phenotype of splenic γ_c^- Ml CD4$^+$ T cells. (A) Splenocytes were stained with CD4 and Vβ6 antibodies and analyzed by FACS. Ml CD4$^+$ T cells are markedly reduced in the absence of γ_c (100-fold decrease in frequency and 250-fold reduction in absolute numbers). (B) Splenocytes were stained with CD4, Vβ6, CD44 and CD62L antibodies and analyzed by FACS. Dot plots show normal CD44 and CD62L expression on gated CD4$^+$ Vβ6$^+$ Ml T cells in the absence of γ_c. (C) Splenocytes were stained with CD4, Vβ6, CD25, CD122 and CD127 antibodies and analyzed by FACS. Dot plots show normal CD127 expression on gated CD4$^+$ Vβ6$^+$ Ml T cells in the absence of γ_c. (D) Forward scatter (FSC), mitochondrial membrane potential (as measured by $DIOC_6$) and H-2D^b expression on splenic CD4$^+$ Vβ6$^+$ Ml T cells show reduced cell size, abnormal survival and lower MHC class I expression in the absence of γ_c. In histogram panels (B–D), γ_c^+ cells (open line) and γ_c^- cells (shaded) are overlaid.

Thus, unlike non-TCR-Tg γ_c^- mice, T cells from Ml γ_c^- female mice had a naive phenotype.

Concerning cytokine receptors, CD127 expression was normal in the absence of γ_c, while naive Ml CD4$^+$ T cells failed to express CD25 or CD122 (Fig. 1C). Consistent with their strongly reduced Bcl-2 levels [23], we found that γ_c^- Ml CD4$^+$ T cells had an abnormal mitochondrial membrane potential as assessed by staining with $DIOC_6$ and were smaller in size than their WT counterparts (Fig. 1D). These results provide an *in vivo* confirmation of the previous report demonstrating that IL-7 can maintain cell size and viability of naive T cells *in vitro* [25]. We further found that γ_c^- Ml CD4$^+$ T cells expressed lower levels of MHC class I molecules (Fig. 1D). These results suggest that γ_c signaling *in vivo* affects the naive T cell phenotype at multiple levels.

We next compared the phenotype of the peripheral cells to the mature CD4 SP thymocytes in γ_c^+ or γ_c^- Ml female mice to assess whether the phenotypes observed were a simple consequence of γ_c deficiency, or whether they resulted from a selective pressure that was imposed in the periphery (Fig. 2A–C). Compared to their γ_c^+ counterparts, CD4 SP thymocytes from γ_c^- Ml female mice bore identical levels of CD4, Vβ6 and CD127 (Fig. 2B). In contrast, γ_c^- CD4 SP thymocytes were smaller in size compared to WT cells and expressed lower levels of H-2D^b (Fig. 2C). These results indicate that γ_c cytokines already begin to play a homeostatic role at the mature CD4 SP thymocyte stage.

Enforced Bcl-2 expression restores peripheral γ_c^- naive CD4$^+$ T cell numbers

We next determined whether the phenotypes associated with γ_c deficiency could be corrected by enforced expression of Bcl-2. Previous studies had shown that Bcl-2 could substantially improve αβ T cell development in mice deficient in IL-7 signaling [13, 14], although it was unclear whether this corrected a defect in the naive cell compartment or improved survival of activated memory T cells that are over-represented in CD127$^{-/-}$ mice [26]. To target Bcl-2 to the naive CD4$^+$ T cell compartment, we generated 'monoclonal' Ml female mice that over-expressed human Bcl-2 in T cells (using the Eμ-2-25 line; [27]) and were either γ_c^+ or γ_c^-.

Tg expression of Bcl-2 had little effect on the absolute number of thymocytes in γ_c^+ or γ_c^- Ml female mice (Fig. 3A), consistent with previous reports on the inability of this Bcl-2 Tg to correct the DN2 block in early thymocyte development in the absence of γ_c [12]. Nevertheless, Bcl-2 dramatically corrected the peripheral T cell defect in these mice (increase of 50-fold), allowing for a near normal number of splenic naive CD4$^+$ T cells despite the absence of γ_c (Fig. 3A, B). This result suggested that poor survival of γ_c^- Ml CD4$^+$ T cells

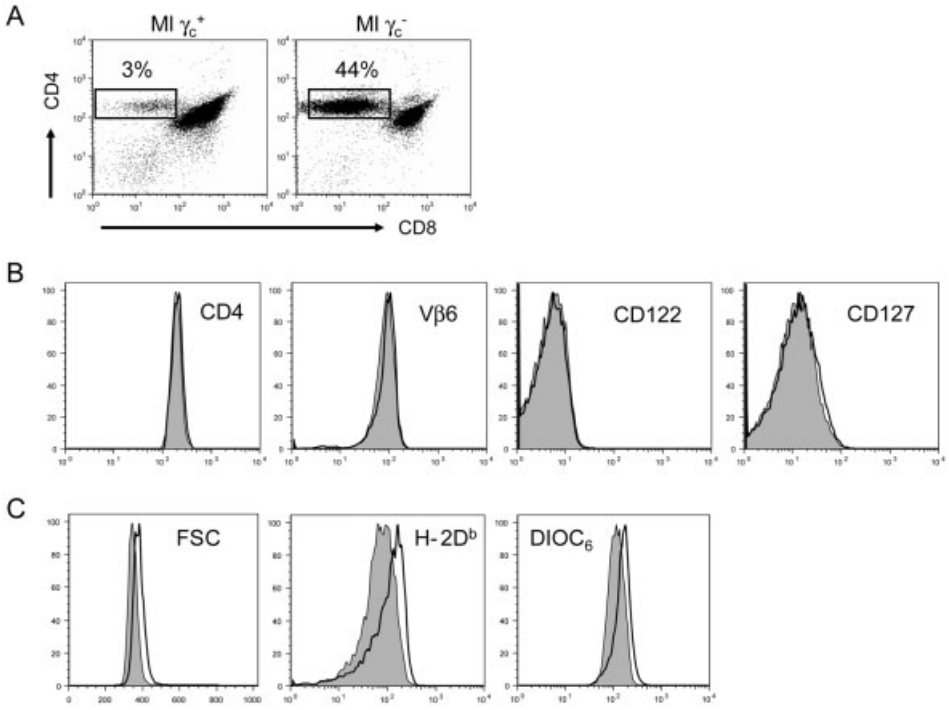

Figure 2. Phenotype of thymic γ_c^- Ml $CD4^+$ T cells. (A) Thymocytes were stained with CD4 and CD8 antibodies and analyzed by FACS. Ml CD4 SP thymocytes are increased in frequency but are present in near normal numbers (see also Fig. 3A). (B) Thymocytes were stained with CD4, CD8, Vβ6, CD122 and CD127 antibodies and analyzed by FACS. Dot plots show normal expression of the indicated markers on gated Ml CD4 SP thymocytes in the absence of γ_c. (C) FSC, mitochondrial membrane potential (as measured by $DIOC_6$) and H-$2D^b$ expression on Ml CD4 SP thymocytes show reduced cell size, abnormal survival and lower MHC class I expression in the absence of γ_c. In histogram panels (B, C), γ_c^+ cells (open line) and γ_c^- cells (shaded) are overlaid.

was a major cause for their reduced cell numbers in the spleen (but not in the thymus). Accordingly, Bcl-2^+ γ_c^- Ml $CD4^+$ T cells had a normal mitochondrial membrane potential (Fig. 3C). Cell surface expression of CD4, Vβ6, CD44, CD62L and CD127 was not modified by Bcl-2 over-expression (Fig. 3C and data not shown).

When cultured *in vitro* in the absence of exogenous γ_c cytokines, Bcl-2^+ Ml $CD4^+$ T cells (either γ_c^+ or γ_c^-) demonstrated enhanced survival compared to non-Bcl-2^+ γ_c^+ Ml $CD4^+$ T cells (data not shown). Interestingly, despite their enhanced survival characteristics *in vivo* and *in vitro*, Bcl-2^+ γ_c^- Ml $CD4^+$ T cells remained small in size. Moreover, these cells continued to have reduced expression of MHC class I (Fig. 3D) and showed a generalized reduction in ribosomal protein transcripts (Supporting Information Table 1). These results confirm and extend previous *in vitro* studies on the role of IL-7 and Bcl-2 in naive T cell homeostasis [21, 25] and demonstrate that Bcl-2-independent pathways are triggered *in vivo* by γ_c cytokines in naive T cells to control cell size and expression of some cell surface proteins.

The ability of exogenous Bcl-2 to rescue a substantial population of γ_c^- naive $CD4^+$ T cells provided the opportunity to formally assess the role for γ_c cytokines in antigen-induced T cell proliferation *in vitro*. IL-2 was initially characterized as 'T cell growth factor' and numerous studies have amply demonstrated the capacity for IL-2 to promote T cell proliferation in culture (reviewed in [28]). We labeled splenocytes from Bcl-2^+ Ml γ_c^+ or γ_c^- female mice with CFSE and cultured the cells in the presence of antigen-presenting cells from female or male CD3ε^- mice. We observed robust proliferation of the Bcl-2^+ Ml $CD4^+$ T cells after antigen stimulation and after several rounds of division, activated T cells up-regulated their expression of several cell surface markers (including CD25, CD44 and CD69) and down-regulated CD62L (Fig. 4A–D and data not shown), while increasing their cell size.

Although a similar profile of antigen-dependent activation was observed in the absence of γ_c, the kinetics showed that γ_c^- cells had a slight delay at day 4 post-stimulation, which was not apparent at day 6 (Fig. 4D). These results indicate that γ_c cytokines were redundant for *in vitro* proliferation provided that their survival was maintained by Bcl-2. Control Ml γ_c^+ $CD4^+$ T cells lacking Bcl-2 showed a similar response (data not shown), suggesting that Bcl-2 over-expression did not dramatically alter the capacity of Ml $CD4^+$ T cells to undergo a normal differentiation program in response to male antigen-presenting cells. Finally, despite their reduced MHC class I expression, Ml γ_c^- $CD4^+$ T cells were not

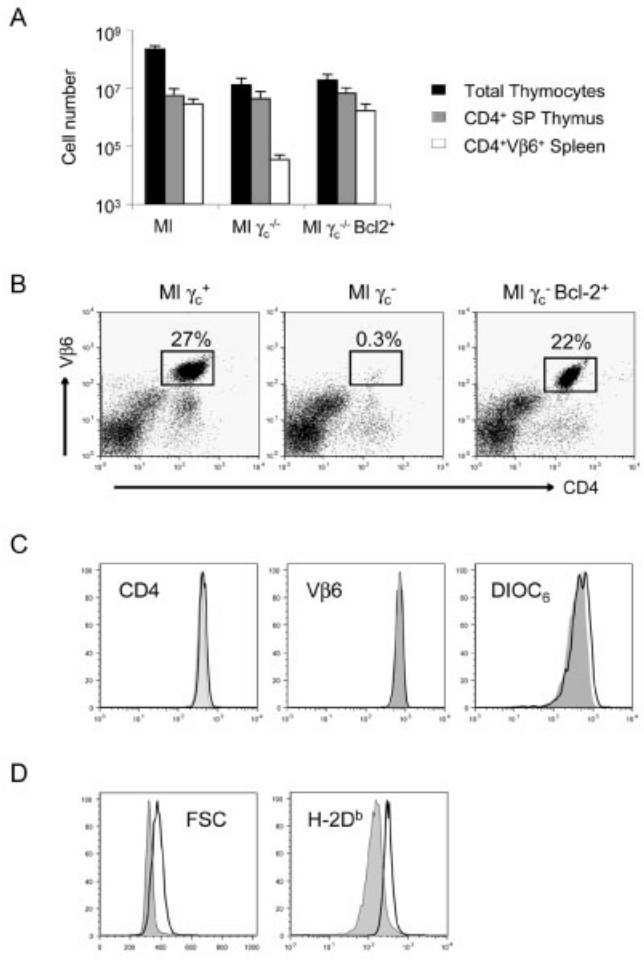

Figure 3. Bcl-2 over-expression rescues peripheral naive γ_c^- Ml $CD4^+$ T cells. (A) Absolute numbers of total thymocytes, CD4 SP thymocytes and splenic $CD4^+$ T cells in Ml γ_c^+, Ml γ_c^- and Ml γ_c^- Bcl-2^+ female mice. Six to 12 mice (4–6 wk of age) of each genotype were analyzed. (B) Splenocytes were stained with CD4 and Vβ6 antibodies and analyzed by FACS. Splenic γ_c^- Ml $CD4^+$ T cells are markedly restored after expression of Bcl-2. (C) CD4 and Vβ6 expression and mitochondrial membrane potential are normal in Ml γ_c^- Bcl-2^+ $CD4^+$ T cells. (D) FSC and H-$2D^b$ expression on splenic $CD4^+$ $V\beta6^+$ Ml γ_c^- Bcl-2^+ T cells show reduced cell size and lower MHC class I expression despite enhanced survival. In histogram panels (C, D), γ_c^+ cells (open line) and γ_c^- cells (shaded) are overlaid.

selectively targeted for elimination by NK cells, despite the presence of the latter in the cultures (data not shown).

Reduced cell size in γ_c^- $CD4^+$ T cells expressing activated PKB

Cytokines appear to regulate naive T cell size in the 'resting' state and can provoke a 'blastogenic' response characterized by increased cell size and enhanced nutrient uptake (reviewed in [29, 30]). Since activation of the intracellular kinase PKB (also known as Akt) in response to growth factors can trigger increases in cell size, metabolism and survival in several cellular systems [29, 30], it has been proposed that IL-7 may act through PKB to mediate its homeostatic effects. Previous studies have shown that an activated PKB Tg could enhance T cell survival *in vivo* [31, 32], although these Tg mice developed autoimmunity and lymphoma, suggesting PKB-mediated transformation. Moreover, conditional deletion of phosphatase and tension homologue deleted on chromosome 10 that counteracts PKB, can bypass the dependence on IL-7 and pre-TCR signals in developing thymocytes [33], although again these mice developed T cell lymphomas. In both these cases, the PKB expression in the peripheral compartment was not restricted to naive T cells. We therefore asked whether a PKB Tg expressed in developing and mature T cells [34] would modify the γ_c deficiency phenotypes that we had observed in naive Ml $CD4^+$ T cells.

We generated monoclonal Ml γ_c^- female mice that were also Tg for Bcl-2 and/or activated PKB. Unlike Bcl-2, the PKB Tg only slightly increased numbers (about threefold) of naive $CD4^+$ T cells in the spleen (Fig. 5A, B, G). Moreover, co-expression of Bcl-2 and PKB Tg did not change the phenotype of naive γ_c^- $CD4^+$ T cells beyond that already observed after enforced Bcl-2 expression (Fig. 5C, D). Ml γ_c^- $CD4^+$ T cells expressing the PKB Tg remained small in size compared to their γ_c^+ counterparts (Fig. 5E, F). Thus, PKB expression in naive T cells is not sufficient to correct the phenotype of γ_c deficiency. In contrast to pharmacological inhibitors that block the PI3K/PKB activation and result in decreased cell size [25, 35], the absence of PKB activation does not appear responsible for the small cell size observed in Ml γ_c^- $CD4^+$ T cells *in vivo*.

Involvement of CD127 but not CD122 in the homeostasis of peripheral $CD4^+$ T cells

In order to identify the γ_c-dependent cytokines that are responsible for $CD4^+$ T cell homeostasis, we generated monoclonal Ml female mice deficient in either CD122 or CD127 and then compared the development of naive $CD4^+$ T cells in these mice with that of their WT counterparts. In the thymus, the absence of CD122 had no discernable effect on thymocyte differentiation, and absolute numbers of total thymocytes and CD4 SP T cells were normal (Fig. 6A). CD4 SP thymocyte size was unaltered (data not shown). In contrast, CD127 deficiency dramatically decreased total thymocyte cell numbers, although positive selection of CD4 SP thymocytes proceeded efficiently (Fig. 6C), with total numbers of CD4 SP thymocytes reaching near normal values (Fig. 6E), similar to what reported previously in the absence of γ_c [23]. These results confirm earlier reports demonstrating that IL-7 (and potentially TSLP)

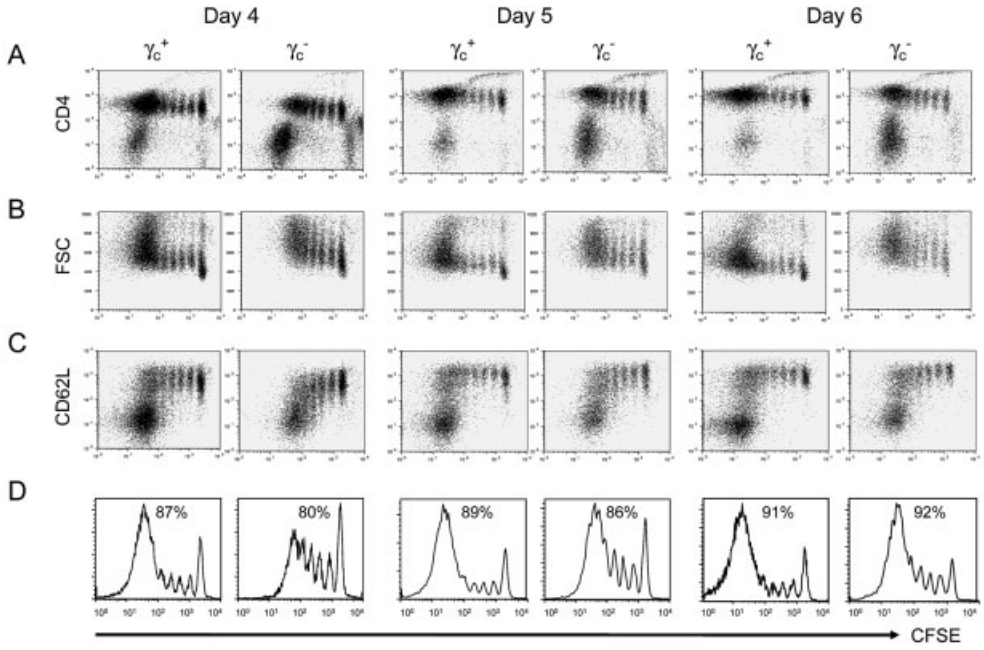

Figure 4. Antigen activation and proliferation of Ml γ_c^- Bcl-2$^+$ T cells *in vitro*. (A) Splenocytes from the indicated mice were labeled with CFSE and cultured in the presence of total splenocytes from male CD3$\varepsilon^{-/-}$ mice for 6 days. At the indicated times, cells were harvested, labeled with CD4, Vβ6 and CD62L antibodies and analyzed by FACS. (B) FSC *versus* CFSE is shown on gated CD4$^+$ Vβ6$^+$ T cells. (C) CD62L expression *versus* CFSE is shown on gated CD4$^+$ Vβ6$^+$ T cells. (D) CFSE dilution profiles on gated CD4$^+$ Vβ6$^+$ T cells are shown. The percentages indicate non-divided CFSE$^+$ cells (right) *versus* divided T cells (left).

but neither IL-2 nor IL-15 are involved in the generation of the SP CD4 thymocyte pool [19].

Deficiency in CD122 had no obvious effect on the peripheral homeostasis of Ml CD4$^+$ T cells (Fig. 6B). Absolute numbers of splenic CD122$^{-/-}$ Ml CD4$^+$ T cells, their size, mitochondrial membrane potential and cell surface phenotype were similar to WT Ml CD4$^+$ T cells (Fig. 6E and data not shown). In contrast, CD127 deficiency resulted in a marked reduction of Ml CD4$^+$ T cells in the spleen; residual CD4$^+$ T cells were small in size and had reduced DIO_6 staining (Fig. 6F). Curiously, cell surface H-2D^b staining was only slightly decreased on splenic CD4$^+$ T cells from CD127$^{-/-}$ Ml female mice (Fig. 6F), suggesting that γ_c cytokines other than IL-7 may be important for this phenotype. Moreover, careful comparison of splenic CD4$^+$ T cell numbers in γ_c^- and CD127$^{-/-}$ Ml female mice showed that the former were significantly reduced compared to the latter (Figs. 3A, 6E; $p<0.05$), consistent with the notion that naive T cell homeostasis depends on γ_c cytokines beyond IL-7.

Previous studies had shown that Bcl-2 could not replace the requirement for IL-7 in the homeostatic expansion of peripheral CD8$^+$ T cells [36]. We assessed the *in vivo* role for IL-7 and Bcl-2 expression for the survival and homeostatic expansion of naive Ml CD4$^+$ T cells using an adoptive transfer approach. WT or Bcl-2$^+$ Ml CD4$^+$ splenic T cells were labeled with CFSE and transferred to alymphoid hosts that were IL-7$^+$ or IL-7$^-$. After 2 wk, the recipients were sacrificed and Ml CD4$^+$ T cells were enumerated and their CFSE profiles analyzed.

Consistent with earlier reports [24], we found that Ml CD4$^+$ T cells underwent two to three rounds of division after transfer to alymphoid recipients, while few T cells were recovered after transfer to IL-7-deficient hosts (Fig. 7). Bcl-2$^+$ Ml CD4$^+$ T cells showed a similar pattern of 'homeostatic' proliferation after transfer to alymphoid hosts as their Bcl-2 Tg$^-$ counterparts. In contrast, Bcl-2$^+$ Ml CD4$^+$ T cells survived well *in vivo* in the absence of IL-7, although they remained undivided (Fig. 7). These observations confirm the role for IL-7 in the survival of peripheral T cells [36] and further demonstrate that IL-7 is required in a Bcl-2-independent fashion for the homeostatic proliferation of naive CD4$^+$ T cells.

Discussion

The roles played by γ_c cytokines in the homeostasis of naive T cells are only partly defined. The use of γ_c^- mice to address this question was hampered by the spontaneous T cell activation that results as polyclonal repertoires with the potential to react with environmental antigens expand in the absence of regulatory T cells [37, 38]. We therefore utilized a model system

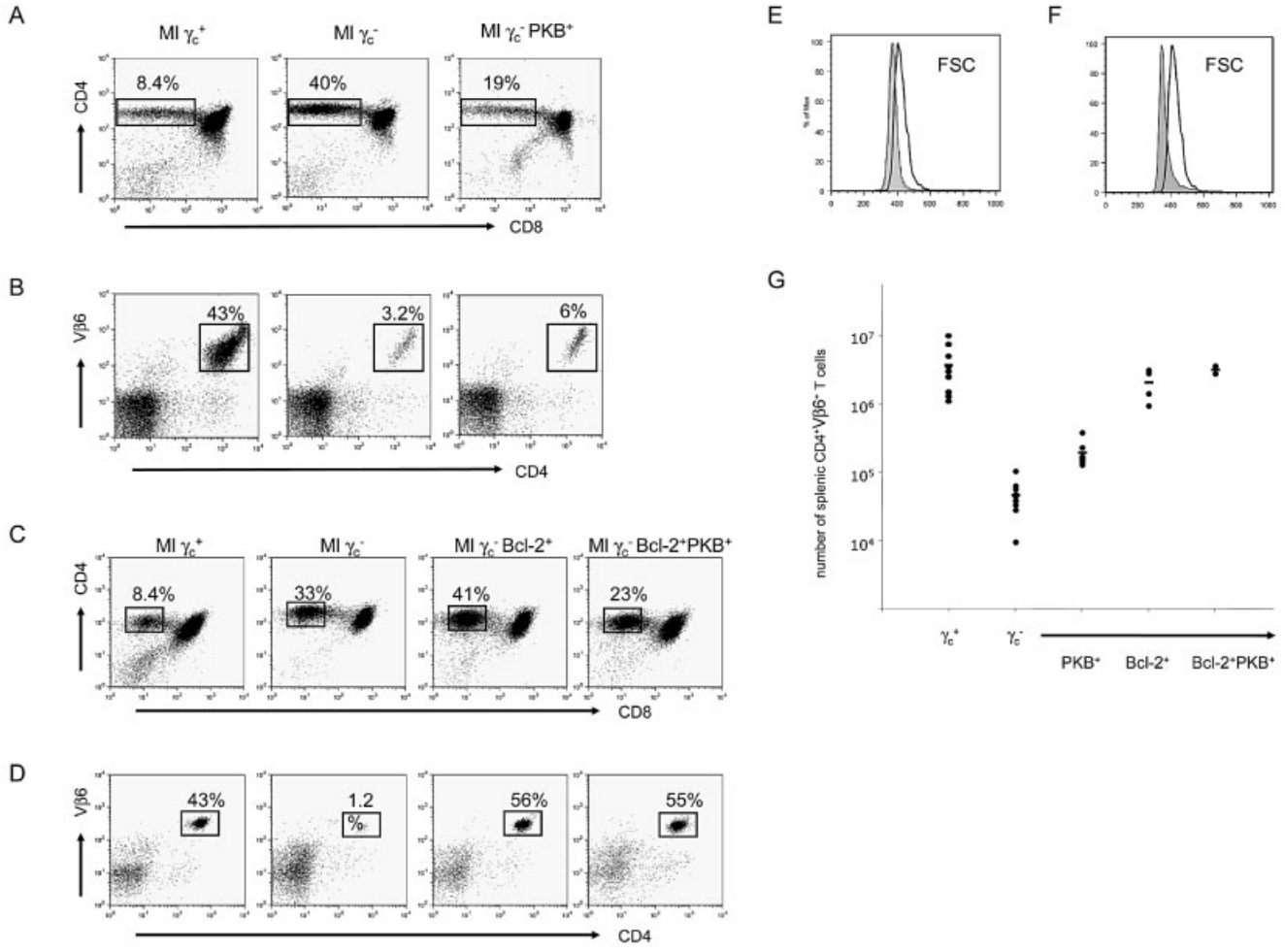

Figure 5. Expression of activated PKB does not affect the peripheral homeostasis of γ_c^- Ml $CD4^+$ T cells. (A, C) Thymocytes were stained with CD4 and CD8 antibodies and analyzed by FACS. (B, D) Splenocytes were stained with CD4 and Vβ6 antibodies and analyzed by FACS. (E) FSC on splenic $CD4^+$ $V\beta6^+$ Ml γ_c^- T cells (shaded) and $CD4^+$ $V\beta6^+$ Ml γ_c^- PKB^+ T cells (dotted line) show reduced cell size compared to γ_c^+ cells (open line). (F) FSC on splenic $CD4^+$ $V\beta6^+$ Ml γ_c^- Bcl-2^+ PKB^+ T cells (shaded) show reduced cell size compared to γ_c^+ cells (open line). (G) Absolute numbers of splenic Ml $CD4^+$ T cells in female mice with the indicated genotype are shown.

based on monoclonal TCR-Tg $CD4^+$ T cells (MI mice) with specificity for a male antigen peptide presented by MHC class II. In female Ml mice, these cells are positively selected, but once exported to the peripheral lymphoid organs show little environmental cross-reactivity. This particular characteristic of Ml-Tg mice was essential, since it allowed us then to generate and analyze γ_c^- Ml $CD4^+$ T cells that maintained a naive phenotype and to assess their properties *ex vivo*.

Using this approach, we were able to show that γ_c^- naïve T cells exhibit decreased survival parameters (mitochondrial membrane potential, reduced expression of Bcl-2), were small in size and had reduced expression of MHC class I molecules. These phenotypic characteristics did not appear to result from a selection process imposed by the peripheral naive T cell niche, since they were also observed in the mature $CD4^+$ thymocyte compartment. By restoring Bcl-2 expression to developing thymocytes and mature γ_c^- naive T cells, we could show that some of the phenotypic characteristics of γ_c deficiency were linked. Bcl-2 over-expression could correct the abnormal mitochondrial potential in γ_c^- $CD4^+$ Ml T cells, and allow these cells to survive *in vitro*. This improved survival property resulted in a rescue of the peripheral naive T cell lymphopenia observed in the absence of γ_c [23]. Nevertheless, the peripheral $CD4^+$ naive T cell numbers were not completely restored (their numbers remained about twofold decreased compared to γ_c WT mice). Possible explanations for this result include either an incomplete thymic rescue by Bcl-2 [12], an additional defect in thymic export and/or the existence of Bcl-2-independent mechanisms for peripheral naive T cell homeostasis.

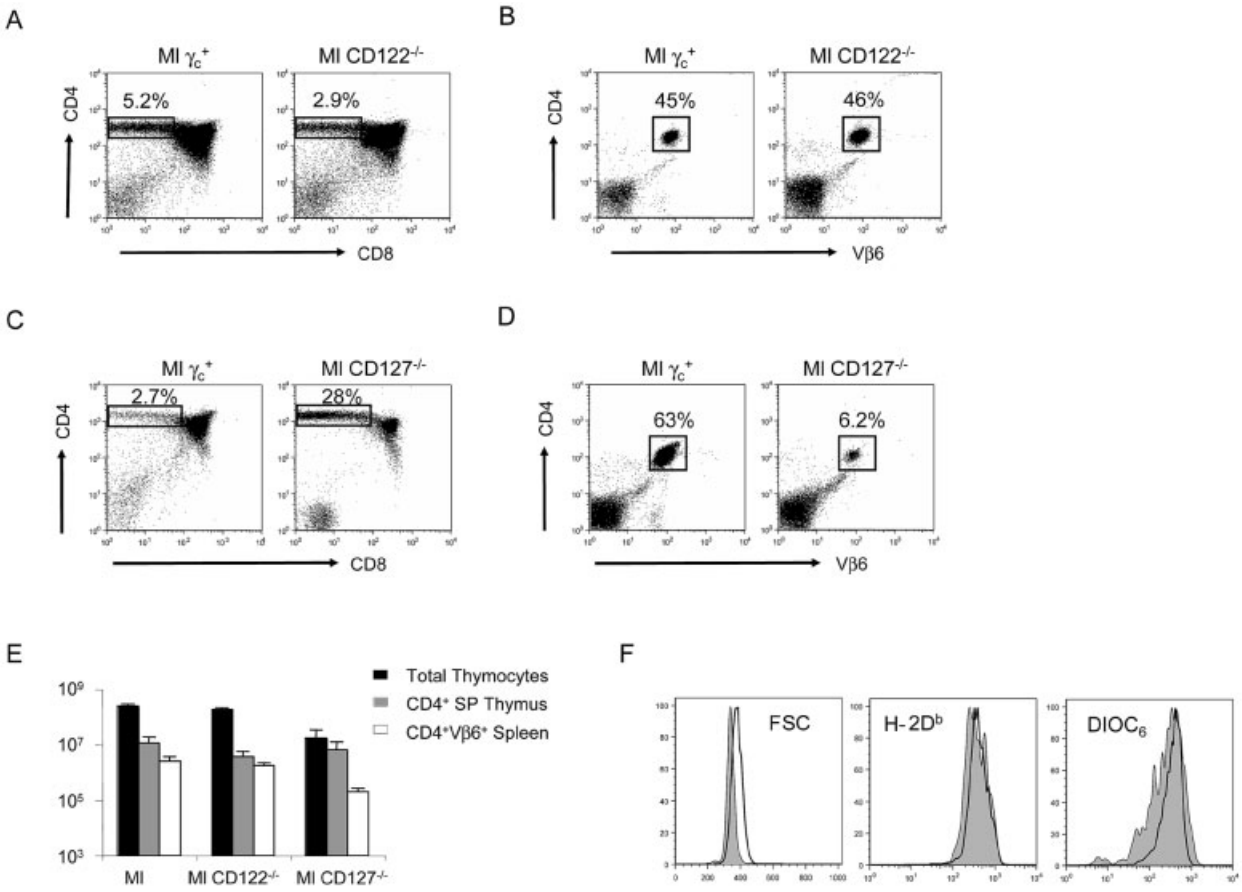

Figure 6. Homeostasis of naive Ml CD4$^+$ T cells requires CD127 but not CD122. (A, C) Thymocytes were stained with CD4 and CD8 antibodies and analyzed by FACS. (B, D) Splenocytes were stained with CD4 and Vβ6 antibodies and analyzed by FACS. (E) Absolute numbers of total thymocytes, CD4 SP thymocytes and splenic CD4$^+$ T cells in Ml γ_c^+, Ml CD122$^{-/-}$ and Ml CD127$^{-/-}$ female mice. Four to eight mice (4–6 wk of age) of each genotype were analyzed. (F) FSC, mitochondrial membrane potential (as measured by $DIOC_6$) and H-2D^b expression on splenic CD4$^+$ Vβ6$^+$ Ml CD127$^{-/-}$ T cells. In histograms, γ_c^+ cells (open line) and CD127$^{-/-}$ cells (shaded) are overlaid.

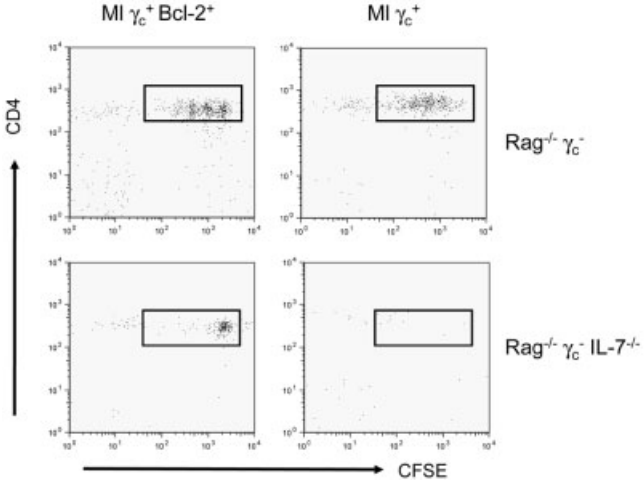

Figure 7. Bcl-2 over-expression allows IL-7-independent survival but not homeostatic proliferation of adoptively transferred Ml γ_c^+ T cells. CFSE-labeled splenocytes from Ml γ_c^+ or Ml γ_c^+ Bcl-2$^+$ female mice were transferred into alymphoid recipients that were IL-7$^+$ (RAG$^{-/-}$ γ_c^-) or IL-7$^-$ (RAG$^{-/-}$ γ_c^- IL-7$^{-/-}$). Two weeks later, splenocytes were harvested and stained with CD4, Vβ6 antibodies and analyzed by FACS. Dot plots show CD4 *versus* CFSE profiles on Vβ6$^+$ gated cells.

Considering the different γ_c-dependent cytokines that are involved in naive T cell homeostasis, a substantial literature exists that provides evidence for a dominant role of IL-7 in naive T cell survival [25, 36, 39]. By comparing female Ml mice deficient in γ_c with those deficient in CD127, we confirm that CD127 ligands are major effectors of naive CD4$^+$ T cell homeostasis. Nevertheless, γ_c cytokines other than IL-7 are likely to be involved, since a stronger reduction in peripheral T cell numbers was observed in the absence of γ_c compared to CD127. Which γ_c-dependent ligands could be involved? We failed to document an effect of CD122 deficiency on naive T cell homeostasis, indicating that IL-2 and IL-15 are redundant in this context. It remains possible that these cytokines become important only in the absence of CD127 although we failed to detect CD122 expression on residual CD4$^+$ T cells in Ml CD127$^{-/-}$ mice (data not shown). Potential roles for IL-4, IL-9 and IL-21 in naive T cell homeostasis therefore remain possible.

Considering Bcl-2-independent mechanisms of γ_c cytokines, we found that γ_c^- Ml CD4$^+$ T cells that have been rescued by Bcl-2 remained small in size. These observations raise questions concerning the potential trophic signals transmitted by γ_c receptors in naive T cells. Previous work from the Thompson laboratory (reviewed in [30]) has enabled the elaboration of a model for cell size regulation. PKB occupies a central role, sitting at the crossroads of the regulation of cell size, metabolism and survival. Activation of PKB results in mTOR-dependent maintenance of glucose and amino acid transporters required for metabolic health of the cell. Nevertheless, the cellular receptor that triggers PKB-dependent trophic effects in naive T cells remains unclear. While IL-7 triggers PKB activation in thymocyte precursors [40] and IL-7 withdraw results in T cell atrophy [40], TCR signals also activate PKB and glucose transporter expression is regulated through the TCR in both thymocyte precursors and peripheral T cells [35, 41]. Direct evidence that PKB is required for naive T cell homeostasis is lacking.

We found that constitutive activation of PKB did not increase the size of naive CD4$^+$ T cells in the absence of γ_c. Therefore, Bcl-2 and PKB activation are not sufficient to maintain naive T cell size and other γ_c-dependent pathways are involved. In the absence of γ_c, naive CD4$^+$ T cells expressed low levels of MHC class I molecules and had reduced expression of ribosomal proteins. These observations suggest a potential mechanism through which γ_c cytokines could exert their effects on cell size. By providing naive T cells with trophic signals that maintain their overall metabolic state (*via* ribosomal activity), γ_c cytokines (including IL-7) may assure protein re-synthesis.

While the restoration of Ml CD4$^+$ naive T cell numbers was impressive, it remains to be seen whether other TCR specificities (when placed in the context of γ_c deficiency) will show the same dependency on Bcl-2. Intrathymically, differentiating CD4 and CD8 SP thymocytes appear to have different requirements for γ_c cytokines [42]. Our preliminary data indicate that Bcl-2 expression can rescue γ_c deficiency in certain TCR-Tg models that select CD8 T cells (OT-I, P14) but not others (HY-CD8) despite clear Bcl-2 expression in the relevant peripheral T cell population (J.P.D., unpublished observations). Gene expression profiling may provide clues to understand the molecular basis for these differences. It is likely that these observations illustrate the concerted actions of TCR-induced signals and γ_c cytokine-induced signals in reaching a minimal threshold required for peripheral naive T cell homeostasis [43].

Materials and methods

Animals

Ml TCR-Tg mice specific for the male antigen HY Dby on the RAG2$^{-/-}$ or RAG2$^{-/-}$/$\gamma_c^{-/-}$ (C57BL/6) background have been described [23]. Mice Tg for an activated PKB [34] or human Bcl-2-Tg mice B6.Cg-Tg$^{(BCL2)25Wehi/J}$ [27] were backcrossed to Ml RAG2$^{-/-}$ or RAG2$^{-/-}$/$\gamma_c^{-/-}$ mice to generate 'monoclonal' Ml Bcl-2$^+$ mice with or without γ_c. CD122$^{-/-}$ mice (B6$^{Il2rbtm1Mak/J}$, JAX) or CD127$^{-/-}$ mice (B6.129S7$^{Il7ratm1Pes/J}$, JAX) were used to generate 'monoclonal' Ml CD122$^{-/-}$ or CD127$^{-/-}$ mice. Mice deficient in RAG2 and IL-7 have been described [20]. Donor and recipient mice were used between 4 and 8 wk of age. Animals were kept under pathogen-free conditions in the animal facilities at the Institut Pasteur and all animal experiments were approved by a local committee and in accordance with French law.

Cell isolation and FACS

Single-cell suspensions from thymus and spleen were prepared as described [20]. Cell suspensions were stained in PBS with 2% FCS during 15 min on ice in the dark. Before staining, cells were treated with purified mouse IgG to block Fc receptors. Monoclonal antibodies conjugated to fluorescein isothiocyanate, phycoerythrin (PE), PE-Cy5.5, peridinin chlorophyll-a protein-Cy5.5, PE-Cy7, allophycocyanin, allophycocyanin-Cy7, Alexa750 or biotin (eBioscience and BD Biosciences) included CD2 (RM2-5), CD4 (GK1.5, RM4-5), Vβ6 (RR4-7), CD25 (PC61), CD44 (1M7), CD62L (MEL-14), CD69 (H1.2F3), CD122 (TMβ1) and CD127 (A7R34). Biotinylated antibodies were revealed with streptavidin-PE-Cy7 or allophycocyanin-Cy7. Dead cells were excluded using TO-PRO3 or Sytox Green (Molecular Probes). FACS acquisitions were performed using Calibur® or Canto® (BD) analytical flow cytometers, and data sets were analyzed using Flowjo® software.

In vitro T cell activation and proliferation

Splenocytes were labeled with 1 μM CFSE as described [20] and the equivalent of 50 000 CD4$^+$ T cells were co-cultured with an equal number of splenocytes from CD3ε$^{-/-}$ male mice in a total volume of 200 μL in RPMI 1640 medium with 10% FCS. Cells were harvested after 4 days and the CFSE dilution profiles of CD4$^+$ T cells assessed by FACS.

Adoptive transfer experiments

Thymocytes from female Ml mice were labeled with CFSE, and the equivalent of 2×10^6 CD4 SP thymocytes was transferred to unconditioned RAG2$^{-/-}$/$\gamma_c^{-/-}$ or RAG2$^{-/-}$/$\gamma_c^{-/-}$/IL-7$^{-/-}$ mice (on the C57BL/6 background) *via* the retro-orbital chamber. Splenocytes were harvested 2 wk later and CFSE$^+$ CD4$^+$ T cells enumerated and analyzed by FACS.

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II. Article 2: γ_c deficiency precludes CD8 T cell memory despite formation of potent T cell effectors

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γ_c deficiency precludes $CD8^+$ T cell memory despite formation of potent T cell effectors

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Abstract

Several cytokines (including IL-2, IL-7, IL-15 and IL-21) that signal through receptors sharing the common γ chain (γ_c) are critical for the generation and peripheral homeostasis of naive and memory T cells. Recently, we demonstrated that effector functions fail to develop in $CD4^+$ T cells that differentiate in the absence of γ_c. In order to assess the role of γ_c cytokines in cell-fate decisions that condition effector versus memory $CD8^+$ T cell generation, we compared the response of $CD8^+$ T cells from γ_c^+ or γ_c^- P14 TCR transgenic mice after challenge with lymphocytic choriomeningitis virus. The intrinsic IL-7-dependent survival defect of γ_c^- naive $CD8^+$ T cells was corrected by transgenic expression of human Bcl-2. We demonstrated that although γ_c-dependent signals are dispensable for the initial expansion and the acquisition of cytotoxic functions following antigenic stimulation, they condition the terminal proliferation and differentiation of $CD8^+$ effector T cells (i.e.: $KLRG1^{high}$ $CD127^{low}$ short-lived effector T cells) via the transcription factor, T-bet. Moreover, the γ_c-dependent signals that are critical for memory T cell formation are not rescued by Bcl2 overexpression. Together, these data reveal an unexpected divergence in the requirement for γ_c cytokines in the differentiation of $CD4^+$ versus $CD8^+$ cytotoxic T lymphocytes.

Introduction

$CD8^+$ T cells are an essential component of the adaptive immune response to pathogens including viruses, bacteria and protozoa. Multiple parameters condition the generation of short-term effector $CD8^+$ T cells and long-term memory $CD8^+$ T cells (1, 2). Upon recognition of pathogen-encoded peptides and appropriate costimulation, $CD8^+$ T cells undergo massive clonal expansion, and activation with profound modifications in their gene expression profile, leading to the differentiation of potent anti-viral effector cells (3, 4). These effector T cells patrol non-lymphoid tissues, and via rapid cytokine secretion and granule exocytosis, eliminate infected cells. The majority of $CD8^+$ effector cells die from apoptosis, mediated by the B cell lymphoma 2 (Bcl-2) family proteins and the cell surface receptor CD95 (5). Immune protection is established by the T cells surviving this 'contraction' phase (6) and maintained by slow basal homeostatic proliferation (7). Memory $CD8^+$ T cells conserve key effector traits and high proliferative potential, thus ensuring rapid protection upon re-infection (8).

In recent years, the cell-fate programming of $CD8^+$ T cells has been a major focus of interest. Indeed, a better understanding of the signals involved in the transition from effector to memory T cells could lead to the design of better vaccination strategies. The IL-7 receptor alpha-chain (CD127) has been proposed as a marker for $CD8^+$ T cells destined to become memory cells (9) and the differential expression of CD127 and the killer cell lectin-like receptor G1 (KLRG1) by activated $CD8^+$ T cells identified two subsets with distinctive cell fates: the $KLRG1^{high}$ $CD127^{low}$ short-lived effector cells (SLEC) and $KLRG1^{low}$ $CD127^{high}$ memory precursor effector cells (MPEC) (10, 11). The strength and duration of the antigenic signal, the $CD4^+$ help received and the cytokine milieu have all been shown to influence the outcome of the $CD8^+$ T cell response (1, 12, 13). However, specific versus redundant roles for

cytokines signaling through the common gamma chain (γ_c) in this cell fate decision programming are still poorly defined.

Interleukin (IL)-2, -7, -15 and -21 share the γ_c receptor chain and its downstream signaling pathway, and influence distinct steps in the $CD8^+$ T cell immune response. The indispensable role for γ_c cytokines in central T cell development, and peripheral T cell homeostasis is well established (7, 14). While IL-7 is vital for the survival and homeostatic proliferation of naive and memory $CD8^+$ T cells (15), IL-2 and IL-15 are essential for the acute proliferation, contraction and cell-renewal capacity of the $CD8^+$ T cells (16-18). Moreover, IL-21 production by $CD4^+$ T cells promotes the cytotoxic function and maintenance of $CD8^+$ effector T cells in the context of chronic viral infections (19, 20). While these studies define roles for γ_c cytokines in $CD8^+$ T cell memory generation, it is unclear at which step of the differentiation process these cytokines impact and what is their importance on the cell-fate decision towards terminal differentiation versus memory generation.

The role for γ_c cytokines in programming effector $CD4^+$ T cells has been previously reported. Using the Marilyn TCR transgenic model of skin graft rejection, we showed that γ_c cytokines condition the progressive differentiation of $CD4^+$ T cells (21). In the absence of γ_c, 'spurious' $CD4^+$ T cells are generated that show an activated phenotype ($CD44^{hi}CD62L^{lo}$) and migrate to the skin, but are unable to elicit graft rejection secondary to severe deficiencies in cytotoxic effector molecules and cytokine production capacities (21). The γ_c-dependent signals involved in programming cytotoxic $CD4^+$ T cells engage both STAT5 and PI3K-dependent pathways (22). Based on these results, we hypothesized an analogous role for γ_c cytokines in the differentiation of $CD8^+$ T cells. However, γ_c cytokines are pleiotropic factors that can play complementary or overlapping roles in the CD8 differentiation process. In order to assess the impact of γ_c cytokines on CD8 T cell programming, we derived TCR transgenic mice on the recombination-activating gene 2 (Rag2) deficient background with or without γ_c.

Furthermore, we corrected the intrinsic survival defect of $\gamma_c^{-/-}$ naïve $CD8^+$ T cells by expression of human Bcl2. This approach allowed us to study the entire $CD8^+$ T cell differentiation program in the absence of γ_c.

Results

Expression of Bcl2 rescues peripheral naive P14 CD8$^+$ T cells in the absence of γ_c

γ_c-deficiency affects not only the survival of naive T cells, but also leads to continuous accumulation of activated T cells in secondary lymphoid organs (23, 24). In contrast, $\gamma_c^{-/-}$ TCR transgenic (Tg) mice on the Rag2 deficient background harbor monoclonal populations of naive T cells, thus providing an approach to study the role of γ_c cytokines during immune responses (21, 25). P14 TCR Tg mice that develop CD8$^+$ $\alpha\beta$ T cells specific for the envelope glycoprotein 33-41 (GP_{33-41}) of the lymphocytic choriomeningitis virus (LCMV) were crossed onto the Rag2$^{-/-}\gamma_c^{+/+}$ or Rag2$^{-/-}\gamma_c^{-/-}$ background. In the absence of γ_c, intrathymic development of P14 CD8$^+$ SP T cells was strongly reduced and cells failed to accumulate in peripheral lymphoid organs (Fig S1A-B). The residual peripheral $\gamma_c^{-/-}$ CD8$^+$ P14 T cells display a naive profile (data not shown) but markedly reduced Bcl-2 levels (Figure S1A) similar to that previously described for γ_c-deficient CD4$^+$ T cells (26). Expression of the human Bcl-2 transgene could rescue the peripheral T cell survival defect in Rag2$^{-/-}\gamma_c^{-/-}$ P14 mice, generating naïve splenic CD8$^+$ T cells with a normal phenotype and number (Figure S1). Bcl-2 transgenic Rag2$^{-/-}\gamma_c^{-/-}$ P14 mice therefore provide an experimental model to assess the importance of γ_c signals in the differentiation of effector and memory CD8$^+$ T cells that elicit anti-viral immunity.

γ_c-dependent cytokines condition the proliferation and terminal differentiation of KLRG1^{high} CD127^{low} SLEC

We adoptively transferred P14 Bcl2 γ_c-competent or γ_c-deficient CD8$^+$ T cells into naive C57BL/6 (Ly5.1) recipients and infected them with LCMV Armstrong. Controls included transfer of P14 CD8$^+$ T cells that did not harbor the Bcl2 transgene. Following antigenic stimulation *in vivo*, both P14 Bcl2 $\gamma_c^{+/+}$ and $\gamma_c^{-/-}$ cells, as well as P14 $\gamma_c^{+/+}$ cells,

proliferated initially with the same kinetics, although the peak of expansion was significantly reduced in the absence of γ_c (50 X 10^6 versus 6 X 10^6 antigen-specific cells respectively, $p<0.0001$) (Fig 1A). Interestingly, the dynamics of activation (assessed by monitoring cell surface markers) was largely unchanged in the absence of γ_c (Fig 1B and S2A). Since recent reports have described two $CD8^+$ T cell subsets (based on the expression of KLRG1 and CD127) with distinct functional properties (10, 11), we analyzed splenocytes of infected mice and found a preferential accumulation of $KLRG1^{low}$ $CD127^{high}$ MPEC over $KLRG1^{high}$ $CD127^{low}$ SLEC in γ_c-deficient $CD8^+$ T cell effectors (Fig 1C-D and S2B). As the transcription factors T-bet and eomesodermin are implicated in CD8 T cell differentiation (27, 28), and as SLEC formation requires T-bet expression (10), we analyzed their expression in activated WT and γ_c-deficient P14 Bcl2 $CD8^+$ T cells. Interestingly, the reduction in the SLEC subset was correlated with decreased T-bet expression in $\gamma_c^{-/-}$ $CD8^+$ T cells (Fig 1E). Furthermore, *Tbx21* and *Klrg1* transcripts were markedly reduced at a single-cell level in $\gamma_c^{-/-}$ $CD8^+$ T cells, while *Eomes* levels remained comparable to γ_c^+ P14 Bcl2 $CD8^+$ T cells (Fig 1F and S2C). Together, our data indicate that γ_c cytokines regulate T-bet expression and thereby condition the generation of $KLRG1^{high}$ $CD127^{low}$ SLEC.

CD8 T cell killing function is unaffected by the absence of the γ_c chain, despite reduced granzyme B levels

We next characterized the impact of these alterations on the functional capacities of $\gamma_c^{-/-}$ P14 Bcl2 $CD8^+$ T cells. As shown in Fig 2A, granzyme B protein levels were strongly decreased in activated $CD8^+$ T cells in the absence of γ_c, while perforin protein levels were unaffected. This granzyme B defect was restricted to $KLRG1^{lo}$ cells (Fig 2A, S3A). Morphological analysis of cytotoxic granules demonstrated that granule size and shape were unchanged in $\gamma_c^{-/-}$ $CD8^+$ T cells although average number of granules per cell was reduced

(Fig 2B). Regarding cytokine production, we found that a similar proportion of $\gamma_c^{-/-}$ $CD8^+$ effector T cells produced IFNγ and TNFα following *in vitro* restimulation as their γ_c^+ counterparts, although a significant increase (2-fold) in triple producers ($IFN\gamma^+$ $TNF\alpha^+$ $IL\text{-}2^+$ cells; p=0.002) were found in the absence of γ_c (Fig 2C and S3B, C).

We next assessed the killing capacity of $\gamma_c^{-/-}$ CD8 T cells. As perforin-deficient (*Pfp*$^{-/-}$) mice show defective CTL and NK cell killing (29), we generated chimeric mice by adoptive transfer of naïve γ_c^+ or $\gamma_c^{-/-}$ P14 Bcl2 $CD8^+$ T cells into *Pfp*$^{-/-}$ recipients. Mice were infected with LCMV, and on day 7, we performed an *in vivo* killing assay using $GP_{33\text{-}41}$-loaded target cells. Surprisingly, $\gamma_c^{-/-}$ $CD8^+$ T cells were as potent killers as their $\gamma_c^{+/+}$ counterparts (Fig 2D). We next determined whether $\gamma_c^{-/-}$ P14 Bcl2 T cells could correct or prevent LCMV-induced hemophagocytic lymphohistiocytosis syndrome in *Pfp*$^{-/-}$ hosts. In this model, dysregulated cytotoxic function in response to LCMV infection leads to subsequent macrophages activation, hypercytokinemia, and multi-organ infiltration, resulting in hepatosplenomegaly, pancytopenia, fever/hypothermia, weight loss and death (30). *Pfp*$^{-/-}$ recipients receiving either γ_c^+ or $\gamma_c^{-/-}$ $CD8^+$ T cells survived equally well through the period following LCMV infection and remained healthy (Fig 2E). Both groups maintained their weight, had stable body temperature (Fig S4A, B) and failed to develop organomegaly or pancytopenia (Fig S4C, D). Together, our data indicate that, despite lower number of $KLRG1^{high}$ $CD127^{low}$ SLEC and decreased granzyme B levels, $\gamma_c^{-/-}$ $CD8^+$ T cells have potent effector functions.

Defective $CD8^+$ T cell expansion due to lack of responsiveness to γ_c cytokines in the late proliferative phase

Since the significant differences in $CD8^+$ T cell numbers at the peak of proliferation could be the result of either abnormal proliferation or increased apoptosis, we analyzed expansion kinetics in the early post-infection period by CFSE-labeling the CD8 T cells before

adoptive transfer and infection. The initial precursor frequency was identical in both groups with ~10% surviving cells after transfer ((31), and data not shown). Furthermore, as shown in Fig 3A, the initial kinetics of T cell proliferation was unaffected in the absence of γ_c, with cells dividing at least 4-5 times in the first three days post infection. Although the percentage of cycling cells is identical at day 5 between both groups, $\gamma_c^{-/-}$ cells proliferated less at day 7, with 2.5-fold fewer cells in the S/G2/M phase of the cell cycle and a 1.3-fold increase in non-proliferating cells (Ki-67^{low}) (Fig 3B). Flow cytometric analysis showed no abnormalities in the apoptotic pathway during the contraction period, with normal expression of Fas, TNFRI, TRAILR, caspase 3 and normal mitochondrial membrane potential (Fig 3C and data not shown). Finally, $\gamma_c^{-/-}$ P14 Bcl2 $CD8^+$ T cells did not demonstrate a survival defect in culture (Figure 3D). Collectively, these results demonstrate the requirement for γ_c-dependent signaling for the late phase of the proliferative response (day 5 to 7) in order to maintain an elevated number of effector $CD8^+$ T cells.

Memory cell generation requires γ_c-dependent Bcl2-independent signals

In order to evaluate whether perturbed differentiation of $\gamma_c^{-/-}$ $CD8^+$ T had an impact on the development of memory T cells, we studied LCMV-infected chimeric mice more than 90 days after infection. Surprisingly, $\gamma_c^{-/-}$ P14 Bcl2 $CD8^+$ T cells were not detected after the contraction phase (from day 13 onward, n>15 mice; Fig 4A), despite the presence of KLRG1^{low} $CD127^{high}$ MPEC at the peak of the response (Fig 1D). We failed to detect $\gamma_c^{-/-}$ P14 Bcl2 $CD8^+$ T cells in the spleen or the bone marrow of chimeric mice 90 days post-infection, and re-infection with LCMV Armstrong did not elicit memory responses from γ_c-deficient P14 Bcl2 $CD8^+$ T cells (Fig 4B and data not shown). These results identify a critical γ_c cytokine-dependent but Bcl2-independent signaling pathway for memory T cell generation.

Discussion

Differentiation of $CD8^+$ T cells from the naive state to the fully competent effector cell stage is a progressive process involving both intrinsic and extrinsic factors. It has been proposed that very early in the immune response, $CD8^+$ T cells are imprinted to become either short-lived terminal effectors or long-lived memory cells (11, 32). This process is influenced by numerous parameters, including the duration of antigen exposure and the presence of costimulatory molecules and soluble factors (IL-2, IL-21) derived from $CD4^+$ T cells (1). Stromal cells also play critical roles by elaborating nutritive factors (including IL-7 and IL-15) that promote T cell survival (33). Collectively, the γ_c cytokines IL-2, -7, -15 and -21 have been implicated in T cell survival, activation, differentiation and memory T cell formation and maintenance (14, 16, 18, 34).

We previously used γ_c-deficient mice to assess the unique and redundant roles for γ_c cytokines in $CD4^+$ T cell differentiation (21). We found that γ_c cytokines (especially IL-7, IL-15) conditioned the progressive differentiation of $CD4^+$ T cells and in the absence of γ_c, activated T cells were generated but essentially lacked effector functions. Our present results demonstrate that many aspects of the antigen-driven $CD8^+$ T cell primary immune response proceed normally in the absence of γ_c, including the initial clonal expansion, the classical phenotypic changes associated with activation and the generation of robust effector capacities. These results clearly demonstrate that the requirements for γ_c cytokines in $CD4^+$ versus $CD8^+$ T cell differentiation are remarkably divergent.

What could account for this difference? The inflammatory cytokine milieu, that can include IL-12/IL-23 and type I/II interferon, has been shown to influence the T cell differentiation process (13, 35). In the LCMV model, type I IFN is abundantly produced and may provide accessory cell-dependent signals that can functionally replace the signals provided by γ_c cytokines via functionally redundant JAK/STAT activation pathways (36, 37).

An alternative explanation would imply that $CD4^+$ T cell differentiation would be more dependent on γ_c cytokines that are elaborated during interactions with accessory cells (DC, stroma) than $CD8^+$ T cells. It is known that $CD4^+$ T cells have a strong requirement for co-stimulatory signals during their differentiation (38) that likely extends to γ_c cytokines (21). In contrast, $CD8^+$ T cell differentiation appears intrinsically programmed following antigen encounter (32, 39) that would obviate requirements for prolonged γ_c cytokine stimulation.

While robust $CD8^+$ T cell differentiation was observed in the absence of γ_c, γ_c-dependent signals were necessary for the transition from effector to memory cell, affecting the differentiation and late proliferation of $KLRG1^{high}$ $CD127^{low}$ short-lived effector cells (SLEC). Furthermore, despite the presence of $Bcl2^+$ $KLRG1^{low}$ $CD127^{high}$ long-lived memory precursors (MPEC), γ_c signals were essential for the generation and maintenance of memory cells. Together, our results define the critical stages for γ_c cytokines in the programming of terminal effector $CD8^+$ T cells and in the Bcl2-independent survival and homeostatic proliferation of memory $CD8^+$ T cells.

Previous studies demonstrated that γ_c-dependent cytokines are important determinants of $CD4^+$ and $CD8^+$ T cell fate, with IL-2, -7, -15 and -21 impacting not only on the differentiation process but also the ability to generate and sustain memory responses (7, 14, 16, 18, 34). In all cases, memory $CD8^+$ T cells were detected, albeit at reduced levels (9, 15, 19, 20, 40-45). This reduced memory formation, however, might have been secondary to reduced T cell survival, as γ_c cytokines promote homeostasis through enhanced expression of anti-apoptotic Bcl2 family members (46, 47). Here we find that in the absence of all γ_c cytokine signals, memory $CD8^+$ T cell formation is completely abolished. Moreover, this γ_c-dependent role in memory formation is Bcl2-independent. These observations suggest that multiple γ_c cytokines condition memory formation through survival-independent mechanisms.

What γ_c cytokines are involved in this process? Antigen-specific memory $CD8^+$ T cells are detected in the absence of IL-2, IL-7, IL-15 or IL-21 (19, 20, 40-42, 44). Moreover, adoptive transfer of P14 $CD122^{-/-}$ cells (lacking IL-2Rβ) generates a pool of memory $CD8^+$ T cells (unpublished results), indicating that a combination of γ_c cytokines are involved. IL-7 and IL-15 have overlapping roles in T cell homeostasis (14, 48, 49) and a similar synergy may operate during memory generation. The downstream targets of IL-21 in memory T cells are poorly defined and could promote memory through distinct pathways.

Triggering of γ_c receptors is linked to enhanced survival. Anti-apoptotic molecules other than Bcl2 are targets of γ_c cytokines, including Mcl-1 that is downstream of IL-7 (50). γ_c-dependent cytokines also regulate transcriptional profiles. One example is the B cell transcription repressor Blimp-1 that is involved in the terminal differentiation of $CD8^+$ T cells (51). Of note, $Blimp1^{-/-}$ CD8 T cells fail to upregulate granzyme B and KLRG1 upon activation (52, 53).

Collectively, our results reveal an unexpected divergence in the requirement for γ_c cytokines in the differentiation of $CD4^+$ versus $CD8^+$ cytotoxic T lymphocytes. Nevertheless, γ_c cytokines remain critical determinants of T cell memory. These observations suggest that selective modulation of γ_c cytokines could impact strongly on immunotherapies and should be taken into account when optimizing vaccine protocols.

Materials and Methods

Mice

CD45.2 Rag2$^{-/-}$ P14 TCR Tg mice (P14; expressing a TCR specific for the LCMV GP_{33-41} epitope) were provided by A. Freitas (Institut Pasteur) and backcrossed onto the C57BL/6 (B6) background. P14 Bcl2^{+} mice with or without the γ_c chain were then produced as previously described (26). P14, P14 Bcl2^{+} and P14 Bcl2^{+} $\gamma_c^{-/-}$ chimeric mice were generated by adoptive transfer of 10^5 MACS-purified naive CD45.2 TCR Tg CD8^{+} T cells into naive CD45.1 B6 recipients 24 hours prior to infection. The recipients were purchased from Charles River (Margate, UK), and were 4-6 weeks old at the time of transfer. Chimeric mice were also generated in naive B6$^{Prf1tm1sdz/J}$ (*Pfp*$^{-/-}$) mice provided by G. De Saint-Basile (Inserm U768, Paris, France). All mice were housed in specific pathogen-free facilities at the Institut Pasteur.

Virus

Stocks of the Armstrong strain of LCMV were plaque purified on Vero cells and grown in BHK-21 cells as described previously (54). Infectious LCMV was quantified by plaque assay. Mice were infected by intraperitoneal injection with 2 X 10^5 PFU of LCMV.

Cell isolation and Flow cytometry analysis

Single cell suspensions from thymus, spleen and bone marrow were prepared and stainings were performed as previously describe (26). Antibodies were purchased from eBioscience and BD Bioscience except for Granzyme B (Caltag), TNFR (Biolegend), T-bet (Santa Cruz Biotechnology) and ultra-avidin-R-phycoerythrin (Leinco). MHC class I peptide tetramers were made and used as described (55). Dead cells were excluded using Live/Dead Fixable Aqua Dead Cell stain kit (Molecular Probes, Invitrogen), except for the *in vitro* survival assay

were propridium iodide (3μM) was used. Data was acquired using a FACSCanto flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Intracellular staining and *In vivo* cytotoxicity assay

Intracellular staining was done on freshly isolated splenocytes according to manufacturer's instructions, with the Fixation and Permeabilisation Kits from eBioscience or BD Bioscience. IFNγ, TNFα and IL-2 quantification were performed after in vitro restimulation with gp33-41 peptide (0.2 μg/ml) in the presence of brefeldin A (10 μg/ml) for 4 hours. For cell cycle analysis, DAPI (Sigma) was added at the time of analysis, after intracellular staining for Ki-67. CFSE labeling was done by incubating cells for 10 min at 37^{o}C with 5 μM CFSE in PBS 2% FCS. *In vivo* cytotoxicity assay (CTL assay) were performed as previously described (54). Briefly, a mixture of 10^7 gp33-41 peptide pulsed (10 μM) CFSE^{high}-labeled (1 μM) and 10^7 non-pulsed CFSE^{low}-labeled (0,1μM) splenocytes was injected IV into Pfp$^{-/-}$ chimeric mice at day 7 post-infection. Peptide-specific cytotoxicity was determined 3 hours later.

Multispectral imaging

Cells were stained by monoclonal antibodies as described above and digital imaging was performed on a multispectral imaging flow cytometer (ImageStream100, Amnis Corporation, Seattle, WA). At least 15,000 – 25,000 cells were imaged for each sample. The data was analyzed using the manufacturer's software (IDEAS, Amnis Corporation). The spot count algorithm included the creation of a sensitive system mask of granzyme B pixels above background. Positive spots were more than one pixel in radius and twice the intensity of the background. Spot counting accuracy was confirmed by manual verification of each image.

Single-cell gene expression analysis

Single-cell sorting was performed on a FACSAria (BD Biosciences). Individual cells were analyzed for the co-expression of mRNAs coding for KLRG1 (*Klrg1*), CD127 (*IL7ra*), T-bet (*tbx21*), eomesodermin (*eomes)* and CD3ε (*Cd3e*), the latter to ensure CD8 sorting specificity as described (54).

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Figure legends

Figure 1. γ_c-dependent cytokines condition the proliferation and terminal differentiation of KLRG1^{high} CD127^{low} SLEC. 10^5 P14, P14 Bcl2 or P14 Bcl2 $\gamma_c^{-/-}$ CD45.2 $CD8^+$ T cells were adoptively transferred into naive CD45.1 mice that were subsequently infected with 2 X 10^5 PFU of LCMV Armstrong. Expansion and contraction of antigen-specific splenocytes were analyzed by flow cytometry at specific time points. (A) Total number of antigen-specific CD8 T cells was calculated based on GP_{33-41} tetramer and CD45.2 congenic marker staining. Data represent the mean ± SEM of six to twelve mice per time point, from three to five experiments (** $p<0.005$, *** $p<0.0005$, NS $p\geq0.05$) (B) Cell surface expression of the indicated molecules by P14 Bcl2 (shaded) and P14 Bcl2 $\gamma_c^{-/-}$ (line) $CD8^+$ T cells at baseline, day 5 and day 7 post-infection. Numbers indicate mean fluorescent intensity for each population (P14 Bcl2 top, P14 Bcl2 $\gamma_c^{-/-}$ bottom). Results are representative of three to five separate experiments (n=7). (C, D, E) Development of short-lived effector cells (SLEC) is compromised in γ_c-deficient cells. (C-D) MPEC and SLEC subsets were analyzed at day 7 post infection based on KLRG1 and CD127 expression. Bar graph represents the results from four separate experiments (mean ± SEM, n=6-12, *** $p<0.0005$, NS $p\geq0.05$). Numbers in dot plots indicate the percentage of each correspondent population from a representative experiment. (E) T-bet expression was determined in P14 Bcl2 (shaded) and P14 Bcl2 $\gamma_c^{-/-}$ (line) $CD8^+$ T cells at day 7. Numbers indicate mean fluorescent intensity. (F) Individual P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) cells were recovered at day 7 post-infection and were tested directly ex vivo for their individual expression of *Klrg1, IL7r, Tbx21* and *Eomes* mRNA. 50 *CD3ε* positive cells were included in the analysis, from four individual mice in two independent experiments. The percentage of tetramer$^+$ CD45.2$^+$ cells expressing the gene is represented (mean ± SEM , *** $p<0.0005$, NS $p\geq0.05$).

Figure 2. CD8 T cell killing function is unaffected by the absence of the γ_c chain, despite reduced granzyme B levels. (A) Day 7 infected P14 Bcl2 (shaded) and P14 Bcl2 $\gamma_c^{-/-}$ (line) $CD8^+$ T cells were analyzed by flow cytometry for cell surface KLRG1 expression and intracellular granzyme B and perforin expression. Flow cytometry plots are representative of eight mice from five independent experiments. Numbers on histograms indicate the mean fluorescent intensity of each population; numbers in dot plots indicate the percentage of cells in each quadrant. (B) Morphology and enumeration of granzyme B (GrzB) spots in $CD8^+$ $CD45.2^+$ cells at day 7 post infection by multispectral imaging. On the left panel, representative images showing cells containing 1, 2, 3, and >4 GrzB spots per cells. Images include bright field (BF), CD8, CD45.2, GrzB and composite of CD45.2 and GrzB (merge). On the right panel, bar graph showing the average distribution of spot counts (mean ± SEM) in P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) $CD8^+$ T cells from >5000 cells per mice analyzed in duplicate (* $p<0.05$, NS $p\geq0.05$). (C) Splenocytes from day 7 LCMV-infected chimeric mice were stimulated with GP_{33-41} peptide and analyzed for IFNγ, TNFα and IL-2 production by intracellular cytokine staining. Bar graph show the average percent cytokine production (mean ± SEM) by P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) $CD8^+$ T cells (n=5-6 per genotype from three independent experiments, NS $p\geq0.05$). (D-E) P14 Bcl2 or P14 Bcl2 $\gamma_c^{-/-}$ $CD8^+$ T cells were adoptively transferred into naive perforin knock-out (*Pfp*$^{-/-}$) mice that were subsequently infected with 2 X 10^5 PFU of LCMV Armstrong. Killing function and development of hemophagocytic lymphohistiocytosis were followed over time. (D) In vivo CTL assay comparing day 8 P14 Bcl2 $\gamma_c^{+/+}$ (filled circle) and P14 Bcl2 $\gamma_c^{-/-}$ (opened circle) $CD8^+$ effector T cells, to infected (filled square) and uninfected/naive (opened square) *Pfp*$^{-/-}$ cells. The individual percent killing over 3 hours represents the combined results of three independent experiments (*** $p<0.0005$, NS $p\geq0.05$). (E) Survival

of *Pfp*$^{-/-}$ (filled square) chimeric mice transferred with P14 Bcl2 (filled circle) and P14 Bcl2 $\gamma_c^{-/-}$ (opened circle) CD8$^+$ T cells.

Figure 3. Defective CD8 T cell expansion is related to lack of responsiveness to γ_c cytokines in the late phase of the proliferative response. (A) CFSE-labeled P14 Bcl2 or P14 Bcl2 $\gamma_c^{-/-}$ CD45.2 CD8$^+$ T cells were adoptively transferred into naive CD45.1 mice that were subsequently infected with 2 X 10^5 PFU of LCMV Armstrong. CFSE incorporation was determined by flow cytometry at day 3 post infection. Numbers indicate the percentage of divided cells. (B-D) P14 Bcl2 and P14 Bcl2 $\gamma_c^{-/-}$ chimeric mice were generated as described in Fig. 2. (B) Five (left panel) and seven (right panel) days post infection, P14 Bcl2 (upper panel) and P14 Bcl2 $\gamma_c^{-/-}$ (lower panel) CD8$^+$ T cells were analyzed by flow cytometry for Ki-67 and DAPI (n=3). Numbers indicate the percentage of cells in each boxed gate. (C) Cell surface expression of the indicated apoptotic-related molecules by P14 Bcl2 (shaded) and P14 Bcl2 $\gamma_c^{-/-}$ (line) CD8$^+$ T cells was analyzed by flow cytometry at day 7 post-infection (n=3). (D) Day 5 post infection, splenocytes were recovered from the appropriate mice and kept in culture for 48 hours. Bar graph shows the corrected percentage of CD8$^+$ CD45.2$^+$ T cells alive based on a propidium iodide staining.

Figure 4. Memory cell generation is established by γ_c-dependent Bcl2-independent signals. P14 Bcl2 and P14 Bcl2 $\gamma_c^{-/-}$ chimeric mice were generated as described in Fig. 2, and were followed longitudinally. (A) The frequency of GP_{33-41}^+ CD45.2$^+$ cells from P14 Bcl2 (filled circle) and P14 Bcl2 $\gamma_c^{-/-}$ (opened circle) CD8$^+$ T cells is evaluated by flow cytometry over a 90 day time course. Results are representative of six to ten mice from three independent experiments. (B) Absolute number of GP_{33-41}^+ CD45.2$^+$ T cells in the spleen and bone marrow of P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) chimeric mice at 90 days post infection (n=4, N.D. not detected).

Supporting Information (SI)

Supplementary Figure 1. Bcl2 over-expression rescues peripheral naive $\gamma_c^{-/-}$ P14 CD8 T cells. (A) Flow cytometry analysis of P14, P14 $\gamma_c^{-/-}$ and P14 Bcl2 $\gamma_c^{-/-}$ thymocytes (left panel) and splenocytes (right panel) using a combination of CD4, CD8, GP_{33-41} tetramer and Bcl2 antibodies. Mouse Bcl2 (black line), isotype control (dotted line) and human Bcl2 (red line) are represented when appropriate. Numbers in dot plots indicate the percentage of each correspondent population. Numbers in histogram indicate mean fluorescent intensity of each population (mouse Bcl2 in black, isotype in grey, human Bcl2 in red). Data is representative of three independent experiments. (B) Absolute numbers of total thymocytes, CD8 single positive (CD8 SP) thymocytes and splenic $CD8^+$ $Tetramer^+$ T cells in P14, P14 $\gamma_c^{-/-}$ and P14 Bcl2 $\gamma_c^{-/-}$ mice. Five to eleven mice (5-9 weeks of age) of each genotype were analyzed. Differences were significant when comparing the three different genotypes in each cellular population (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$). (C) Cell surface expression of the indicated molecules by P14 Bcl2 (shaded) and P14 Bcl2 $\gamma_c^{-/-}$ (line) CD8 T cells at baseline. Results are representative of at least three separate experiments.

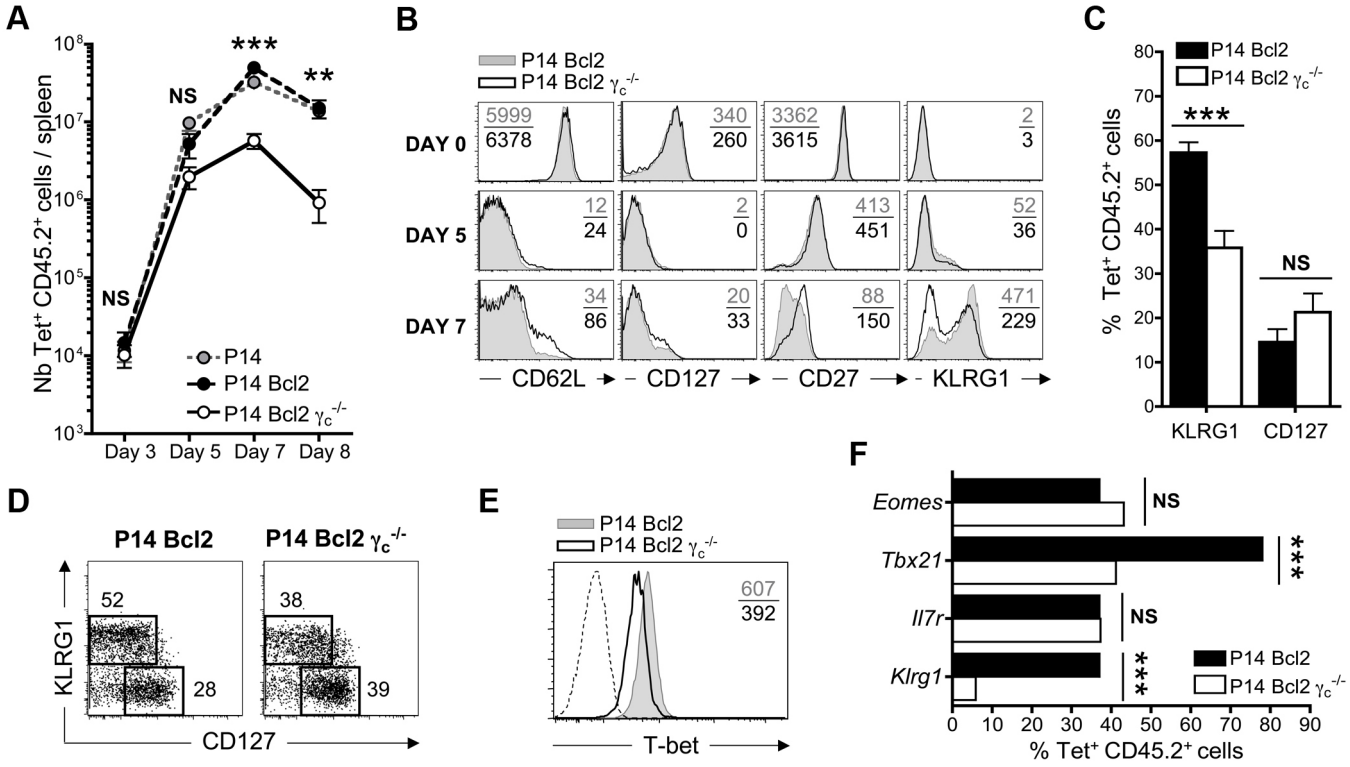
Supplementary Figure 2. γ_c-dependent cytokines are dispensable for the acquisition of numerous activation markers, but are essential in the differentiation of SLEC. 10^5 P14 Bcl2 or P14 Bcl2 $\gamma_c^{-/-}$ CD45.2 CD8 T cells were adoptively transferred into naive CD45.1 mice that were subsequently infected with 2 X 10^5 PFU of LCMV Armstrong. (A) Cell surface expression of the indicated molecules by P14 Bcl2 (shaded) and P14 Bcl2 $\gamma_c^{-/-}$ (line) CD8 T cells at day 7 post-infection. Results are representative of three to five separate experiments (n=7). (B) MPEC and SLEC subsets were analyzed at day 7 post infection, and are represented in bar graphs by the mean fluorescent intensity of KLRG1 and CD127 from

four separate experiments (mean ± SEM, n=6-12, *** $p<0.0005$, NS $p\geq0.05$). (C) Individual P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) splenocytes were recovered at day 7 post-infection and were tested directly ex vivo co-expression of the indicated mRNAs. Each horizontal row represents the pattern of gene expression in the same single cell; gene expression is indicated in black, negative results are shown in white.

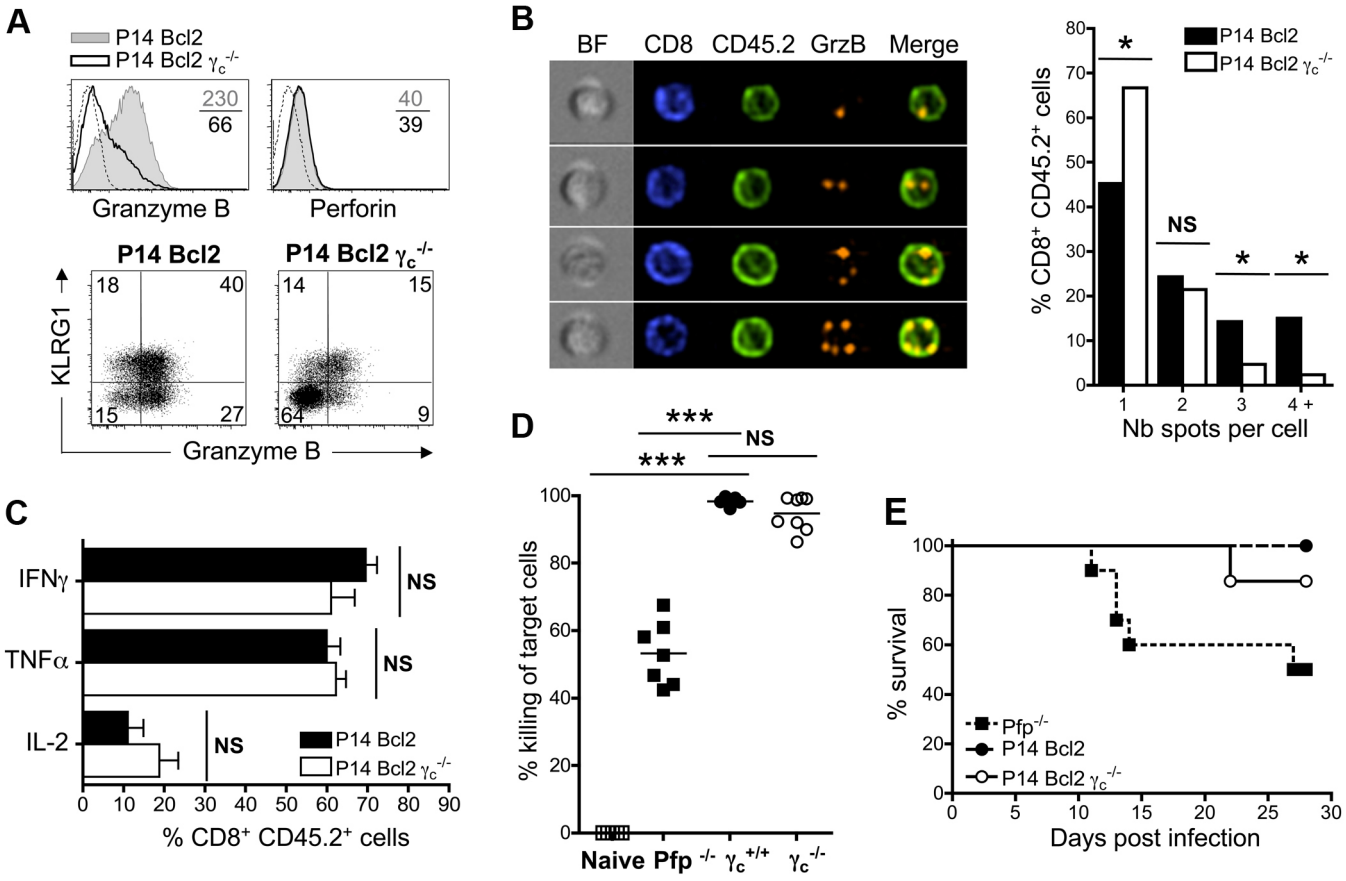
Supplementary Figure 3. γ_c-deficient CD8 effector T cells present decreased granzyme B levels but conserve the ability to secrete cytokines upon restimulation. (A) Day 7 infected P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) CD8 T cells were analyzed by flow cytometry for cell surface KLRG1 expression and intracellular granzyme B. Bar graph represent the percentage (left panel) and mean fluorescent intensity (right panel) of granzyme B^+ cells in antigen-specific $KLRG1^{high}$ and $KLRG1^{low}$ population (mean ± SEM, n=4, * $p<0.05$, NS $p\geq0.05$). (B-C) Splenocytes from day 7 LCMV-infected chimeric mice were stimulated with GP_{33-41} peptide and analyzed for cytokine production by intracellular staining. (B) Bar graph shows the average mean fluorescent intensity of each cytokine by P14 Bcl2 (black bars) and P14 Bcl2 $\gamma_c^{-/-}$ (white bars) CD8 T cells (n=5-6 per genotype from three independent experiments, NS $p\geq0.05$). (C) Cytokine production presented by flow cytometry plots from a representative experiment; numbers indicate the percentage of cells in each correspondent quadrant. Bar graph represents the corresponding percentage of cells secreting only IFNγ, IFNγ and TNFα, or simultaneously IFNγ, TNFα and IL-2 for each genotype (mean ± SEM, * $p<0.05$, ** $p<0.005$, NS $p>0.05$).

Supplementary Figure 4. γ_c-deficient CD8 T cells hinder the development of hemophagocytic lymphohistiocytosis in perforin knock-out mice. P14 Bcl2 or P14 Bcl2 $\gamma_c^{-/-}$ CD8 T cells were adoptively transferred into naive perforin knock-out ($Pfp^{-/-}$) mice that

were subsequently infected with 2 X 10^5 PFU of LCMV Armstrong. (A) Mean body weight and (B) mean core body temperature of $Pfp^{-/-}$ (filled square) chimeric mice transferred with P14 Bcl2 (filled circle) and P14 Bcl2 $\gamma_c^{-/-}$ (opened circle) CD8 T cells. The data is representative of the mean ± SEM for six to ten mice per group. (C) Spleen (left panel) and liver (right panel) weight relative to body weight at day 7 post infection in P14 Bcl2 (black bars), P14 Bcl2 $\gamma_c^{-/-}$ (white bars) chimeric mice and non-chimeric $Pfp^{-/-}$ (grey bars) mice (n=4-6). Differences are not significant between P14 Bcl2 and P14 Bcl2 $\gamma_c^{-/-}$ groups but each of them is statistically different from the $Pfp^{-/-}$ control group (*$p<0.05$, NS $p\geq0.05$). (D) Hemoglobin level, platelet count, lymphocyte count and neutrophil count at day 12 post infection in P14 Bcl2 (black bars), P14 Bcl2 $\gamma_c^{-/-}$ (white bars) chimeric mice and non-chimeric $Pfp^{-/-}$ (grey bars) mice. Grey shaded area represents the normal values for naive $Pfp^{-/-}$ control mice (mean ± SEM). Differences are not significant between P14 Bcl2 and P14 Bcl2 $\gamma_c^{-/-}$ groups but each of them is statistically different from the $Pfp^{-/-}$ control group (** $p<0.005$, *** $p<0.0005$, NS $p\geq0.05$).



Decaluwe et al. Fig 1



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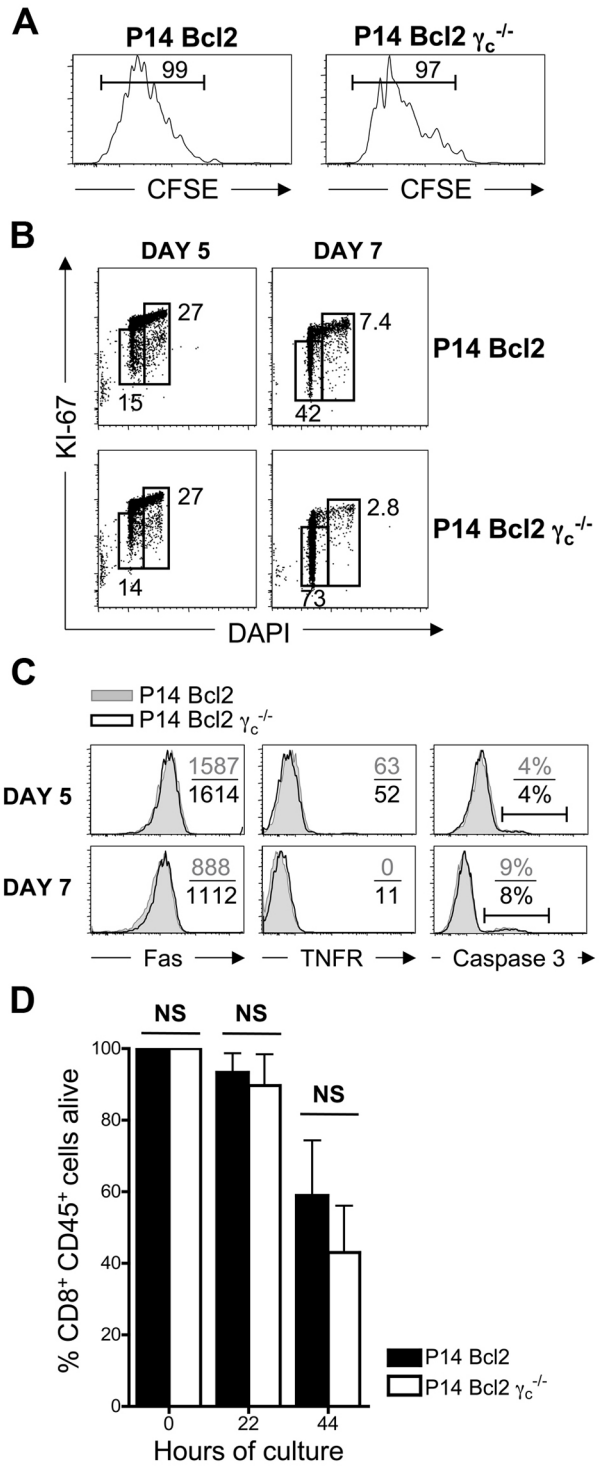

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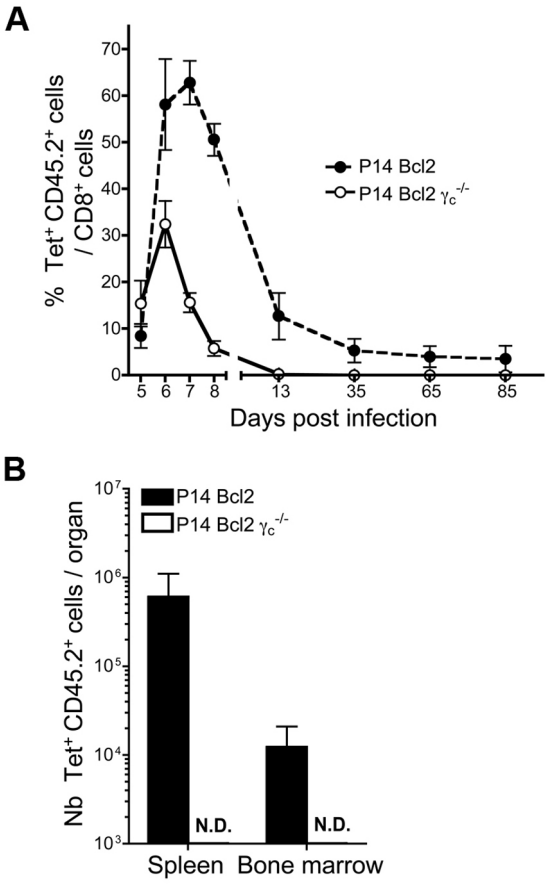

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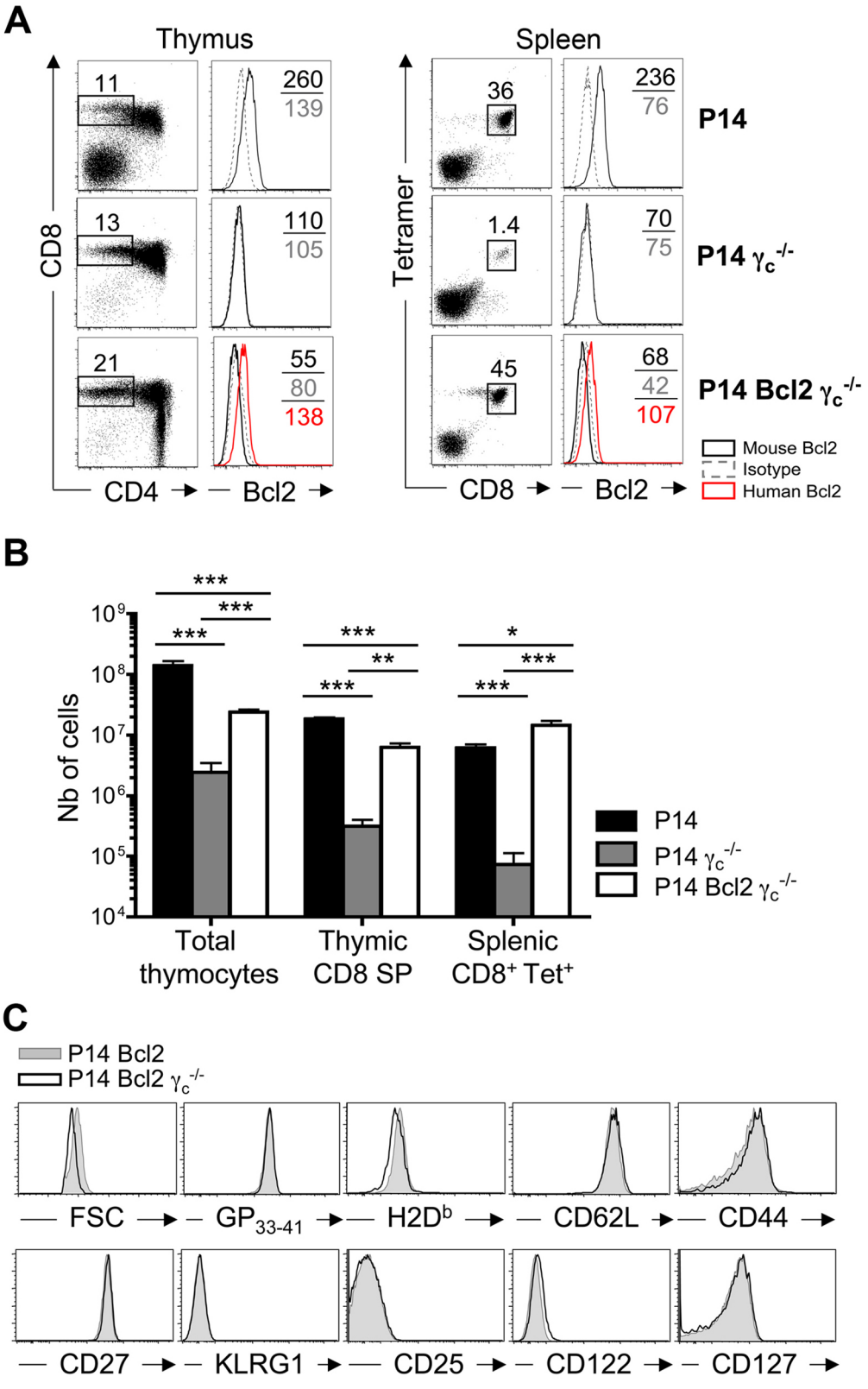

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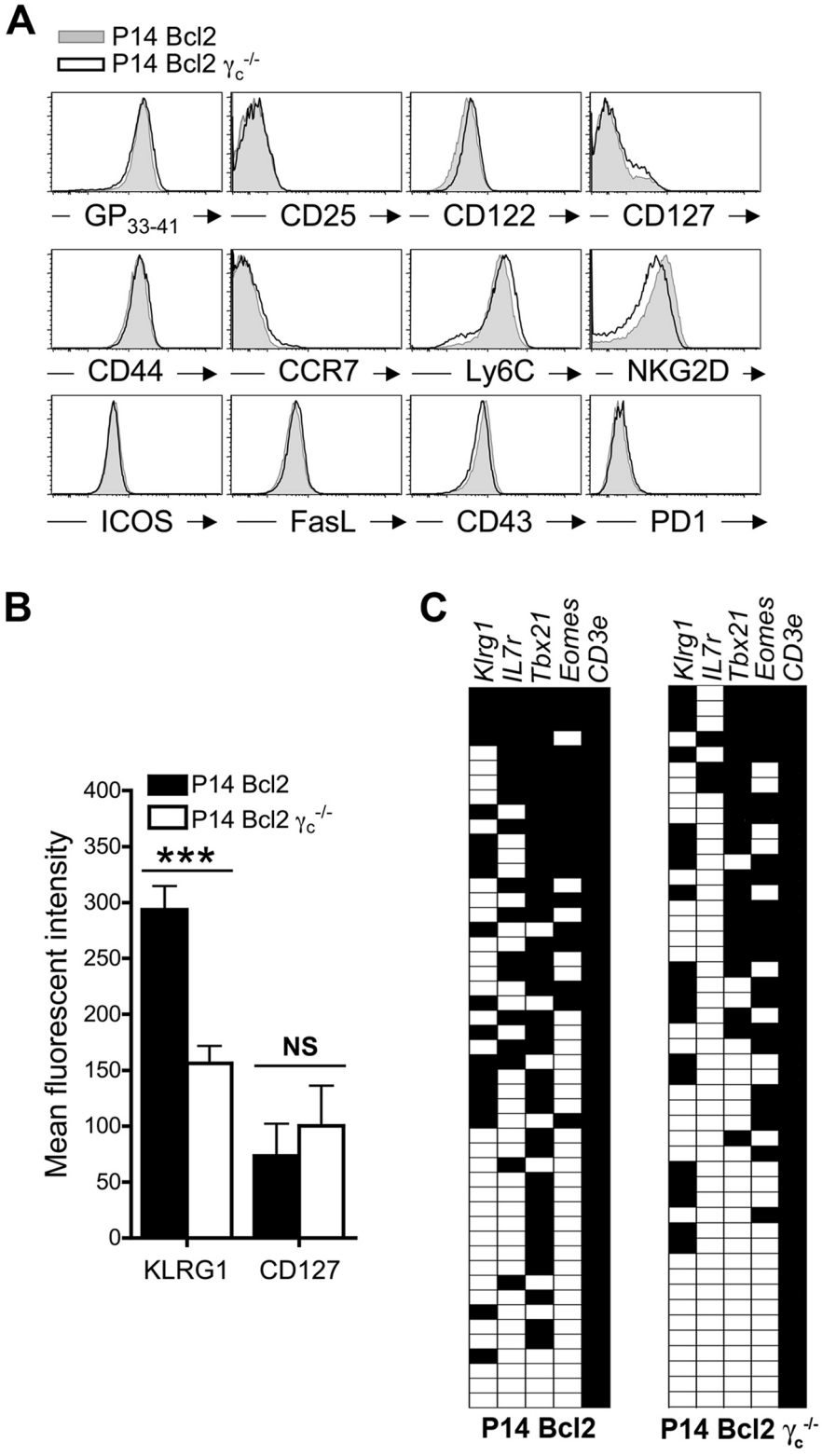

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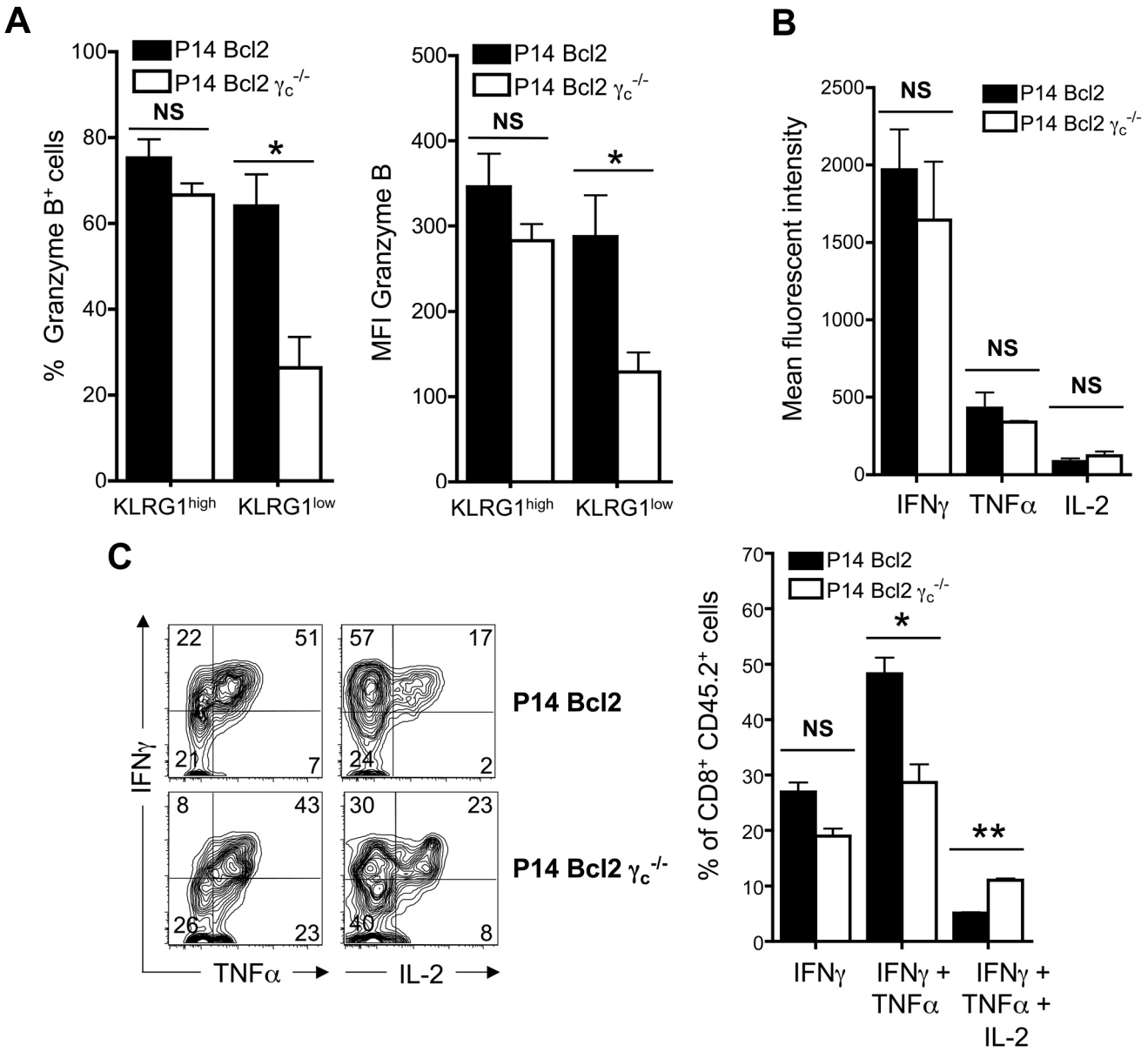

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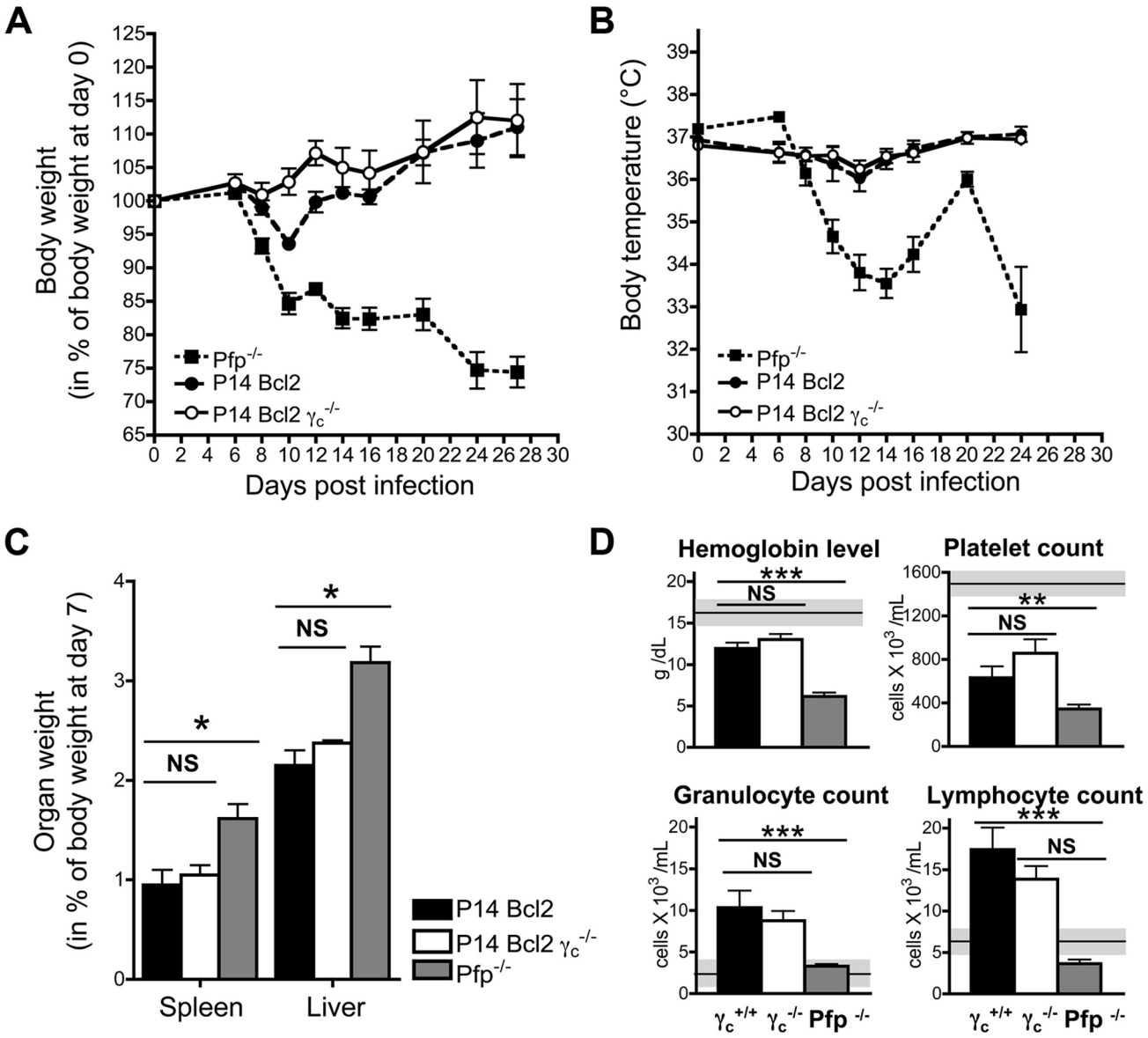

Decaluwe et al. Fig S4

III. Article 3: Epitope specificity and relative clonal abundance do not affect CD8 T cell differentiation patterns during lymphocytic choriomeningitis virus infection

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Epitope Specificity and Relative Clonal Abundance Do Not Affect CD8 Differentiation Patterns during Lymphocytic Choriomeningitis Virus Infection▽

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To evaluate the impact of immunodominance on CD8 T-cell properties, we compared the functional properties of dominant and subdominant populations in the response to lymphocytic choriomeningitis virus (LCMV). To improve functional discrimination, in addition to the usual tests of phenotype and function, we used a sensitive technique that allows the screening of all CD8 effector genes simultaneously in single cells. Surprisingly, these methods failed to reveal a major impact of clonal dominance in CD8 properties throughout the response. Aiming to increase clonal dominance, we examined high-frequency transferred P14 T-cell receptor transgenic (TCR Tg) cells. Under these conditions LCMV is cleared faster, and accordingly we found an accelerated response. However, when Tg and endogenous cells were studied in the same mice, where they should be subjected to the same antigen load, they showed overlapping properties, and the presence of P14 cells did not modify endogenous responses to other LCMV epitopes or a perturbed immunodominance hierarchy in the memory phase. Using allotype-labeled Tg cells, we found that during acute infection up to 80% downregulated their TCR and were undetectable by tetramer binding, and that tetramer-negative and tetramer-positive cells had very different features. Since Tg cells are not available to evaluate immune responses in humans and, in many cases, are not available from the mouse, the tetramer-based evaluation of early immune responses in most situations of high viremia may be incomplete and biased.

The lymphocytic choriomeningitis virus (LCMV)-induced immune cell response in mice is particularly impressive in its breadth, since at the peak of the response ~90% of activated splenic $CD8^+$ T cells are directed against 28 defined epitopes in H-2^b mice (23, 24, 27). The immunodominance hierarchy then observed may be determined by a variety of parameters, including epitope prevalence, antigen processing and/or its binding affinity to major histocompatibility complex (MHC), T-cell precursor frequency and/or recruitment, and T-cell receptor (TCR) affinity and avidity (55). However, the extent to which this immunodominance guides functional performance still is unknown. Related to this issue is the question of whether the information generated from studies involving artificially induced immunodominance by the adoptive transfer of TCR transgenic (Tg) cells at a high precursor frequency can be generalized to endogenous cells, which are present in small numbers (10^{-4} to 10^{-5}) and consist of polyclonal T-cell subpopulations with different avidities. It was suggested recently that $CD8^+$ TCR Tg cells originating from precursors introduced at unnaturally high frequencies exhibit altered differentiation during infection, as they were shown to reexpress CD62L and interleukin-7R (IL-7R) much sooner than endogenous cells (2, 26). However, it remains controversial whether these findings reflect, as suggested, major differences in differentiation pathways (2, 26) or whether the observed dissimilarities are due to differences in differentiation kinetics (35).

Cytotoxic effector $CD8^+$ T cells generated in various infectious models traditionally were regarded as uniform populations that could secrete gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) upon in vitro restimulation and exert cytotoxic effects (12). However, the current methods used to study CD8 function during immune responses have several limitations. Cells producing cytokines usually are not detected directly ex vivo, because these proteins, once produced, are immediately secreted into the environment and do not accumulate inside the cell in amounts sufficient to be visualized by intracellular staining. Therefore, cytokine production currently is detected after in vitro restimulation, but under these conditions antigen-experienced cells from a normal response (where cells are not tolerized) all score very similarly, i.e., it is no longer possible to distinguish the expansion phase, effector peak, or memory cells' cytokine expression capacities; these tests similarly identify all antigen-specific cells throughout the

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response (29). Concerns were raised that the in vitro restimulation necessary for revealing many functional traits could result in the erroneous overestimation of the number and quality of effector cells present in situ at any given time. Indeed, it was shown that in vitro restimulation could induce major alterations in ex vivo readouts: IFN-γ mRNA expression frequencies of 10% evaluated ex vivo were shown to increase to 90% (47), and TNF-α expression increased from <1 to 100% after a 4-h peptide stimulation (31). These differences may be due to the organ's three-dimensional structure, which significantly modifies CD8 responses (38). In addition, cytokine secretion greatly depends on the strength of stimulation (16, 37). Therefore, the in vitro environment may fail to reproduce the in vivo cell interactions, the peculiar inflammatory environment induced by the infection, and the local amount of pathogen-derived peptides.

To monitor CD8 differentiation as it unfolds in vivo, we recently developed a sensitive reverse transcription-PCR (RT-PCR) method capable of measuring the expression of up to 20 genes simultaneously in the same cell without further in vitro manipulation. We showed that this method allows a much better discrimination of cell properties throughout the immune response compared to that of more conventional approaches (28, 32, 33). We could discriminate very different cytokine mRNA expression profiles at different phases of the response. These and other gene expression profiles predicted very different functional properties of CD8 T cells in early expansion, response peak, or memory phase that were confirmed by in vivo functional tests. Notably, we also found that the coexpression frequency of mRNAs coding for perforin and granzyme B in the same cell directly predicted CD8 T cells' cytotoxic capacity (32).

As the approach described above provided us with a more detailed analysis of the behavior of $CD8^+$ T cells during immune responses, we applied it together with other more conventional approaches to study the influence of clonal dominance in the behavior of CD8 T cells after infection. We studied endogenous cells responding to immunodominant (NP396 and GP33) and subdominant (GP276) LCMV epitopes and found they had similar properties, suggesting that the infectious environment rather than TCR specificity or relative clonal abundance had the major influence in shaping T-cell properties. To amplify differences in relative clonal abundance, we further compared high-frequency transferred TCR Tg cells specific for the GP33 epitope (P14) to the endogenous cells recognizing the same or other LCMV peptides. Surprisingly, we found that previously reported differences in Tg behavior (2) could be fully explained by differences in the response kinetics, since they were not found when Tg and endogenous cells were studied in the same mouse. Moreover, P14 transfers did not modify the endogenous response to other LCMV epitopes or the immunodominance hierarchy in the memory phase. Finally, in these adoptive transfer studies we could monitor the transferred Tg population by both allotype labeling and GP33 tetramer binding. We found that during the expansion phase, a substantial fraction of allotype-positive Tg cells downregulated TCR expression and could not be recognized by tetramer binding, and that tetramer-negative (tet^{neg}) and tetramer-positive (tet^{pos}) cells had very different properties. These results reveal that the evaluation of the early immune response in normal individuals by tetramer binding is incomplete and may be very biased. Thus, TCR Tg cells, because of their ease of detection by allotype markers, may provide the only means of accurately characterizing the entire spectrum of activated CD8 T cells in the early stages of the immune response.

MATERIALS AND METHODS

Mice. CD45.2 $Rag2^{-/-}$ P14 TCR Tg mice (P14) expressing a TCR specific for LCMV epitope GP33-41 (GP33) and backcrossed onto the C57BL/6 (B6) background were bred at the Centre de Distribution, Typage et Archivage (CDTA, Orléans, France). B6.CD45.1 and B6.CD45.2 mice were purchased from Charles River (Margate, United Kingdom) and the Jackson Laboratory (Bar Harbor, ME). Animal studies were carried out according to United Kingdom Home Office regulations or the University of Massachusetts Medical School, Department of Animal Medicine, regulations and were approved by the site ethical review committee.

Antibodies, MHC class I (MHC-I) tetramers, and other reagents. Labeled antibodies to CD8, CD45.2, CD45.1, TCRαβ, CD69, CD3, Ly6C, CD25, CD27, CD127, CD44, CD122, KLRG1, IFN-γ, TNF-α, and IL-2 and isotype-matched control antibodies were from either BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Granzyme B was from Caltag. GP33-41 H-$2D^b$ (GP33), NP396-404 H-$2D^b$ (NP396), and GP276-286 H-$2D^b$ (GP276) tetramers were obtained from Beckmann Coulter (Marseille, France). 5-(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR).

Viral growth and titration and infection of mice. LCMV strain Armstrong (clone 5.3b) was grown in BHK-21 cells, and infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (9). B6 mice were infected intraperitoneally with 2×10^5 PFU of LCMV Armstrong. Some animals were inoculated with 5×10^3 or 5×10^5 TCR Tg cells (prepared from the lymph nodes of P14 mice) 1 day prior to infection.

CFSE labeling, intracellular staining, and in vivo cytotoxicity assays. CFSE labeling was done by incubating cells for 10 min at 37°C with 1 μM CFSE in RPMI medium. Cells were labeled with CFSE by incubation for 10 min at 37°C with 1 μM CFSE in RPMI. For intracellular cytokine staining, splenocytes from LCMV-infected mice were incubated without peptide or with 0.2 to 0.4 μg/ml NP396, GP33, or GP276 peptide for 5 h in the presence of 10 μg/ml of brefeldin A, and then cytokine levels were determined. Granzyme B staining was performed without restimulation. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. In vivo cytotoxicity assays were performed as previously described (4). Briefly, a mixture of 10^7 GP33 or NP396 peptide-pulsed (1 μM) CFSE-labeled and 10^7 nonpulsed nonlabeled splenocytes was injected intravenously into LCMV-infected or control mice at 8 and 60 days postinfection. Peptide-specific cytotoxicity was determined in the spleen 12 h later and was calculated using the following formula: $100 - \{100 \times [(\%\text{ peptide pulsed infected}/\%\text{ peptide nonpulsed infected})/(\%\text{ peptide pulsed control}/\%\text{ peptide nonpulsed control})]\}$.

Single-cell purification and gene expression analysis. The purification and single-cell sorting of CD8 T cells was described previously (32). P14 cells were distinguished from endogenous GP33-specific cells by the expression of a congenic marker. Each individual cell was analyzed for the coexpression of mRNAs coding for TGF-β (*Tgfb1*), TNF-α (*Tnf*), IL-2 (*Il2*), IFN-γ (*Ifng*), perforin (*Prf1*), granzyme A (*Gzma*), granzyme B (*Gzmb*), FasL (*Fasl*), and CD3ε (*Cd3ε*), the latter to ensure CD8 sorting specificity. The accuracy and efficiency of the method were described previously (33).

Immunosuppression protocol. Two months after LCMV infection, mice were depleted of T cells by the intraperitoneal injection of 500 μg of anti-CD8α antibody (clone 53.6.7) and 500 μg of anti-Thy1.2 antibody (clone 30H12) twice per week for five consecutive weeks, and lung, lymph nodes, spleen, kidney, testes, brain, liver, and bone marrow were harvested for the determination of virus titers. Virus titers in spleen, serum, or other tissues were determined by plaque forming (49).

Statistical analysis. Associations or dissociations between the pattern of expression of different genes and differences in the expression of individual genes between different populations of cells were analyzed using a two-tailed Fisher's exact test. A P value of <0.05 was considered statistically significant.

Gene nomenclature. Abbreviations used for mRNAs were those recommended by the International Committee on Standardized Genetic Nomenclature for Mice.

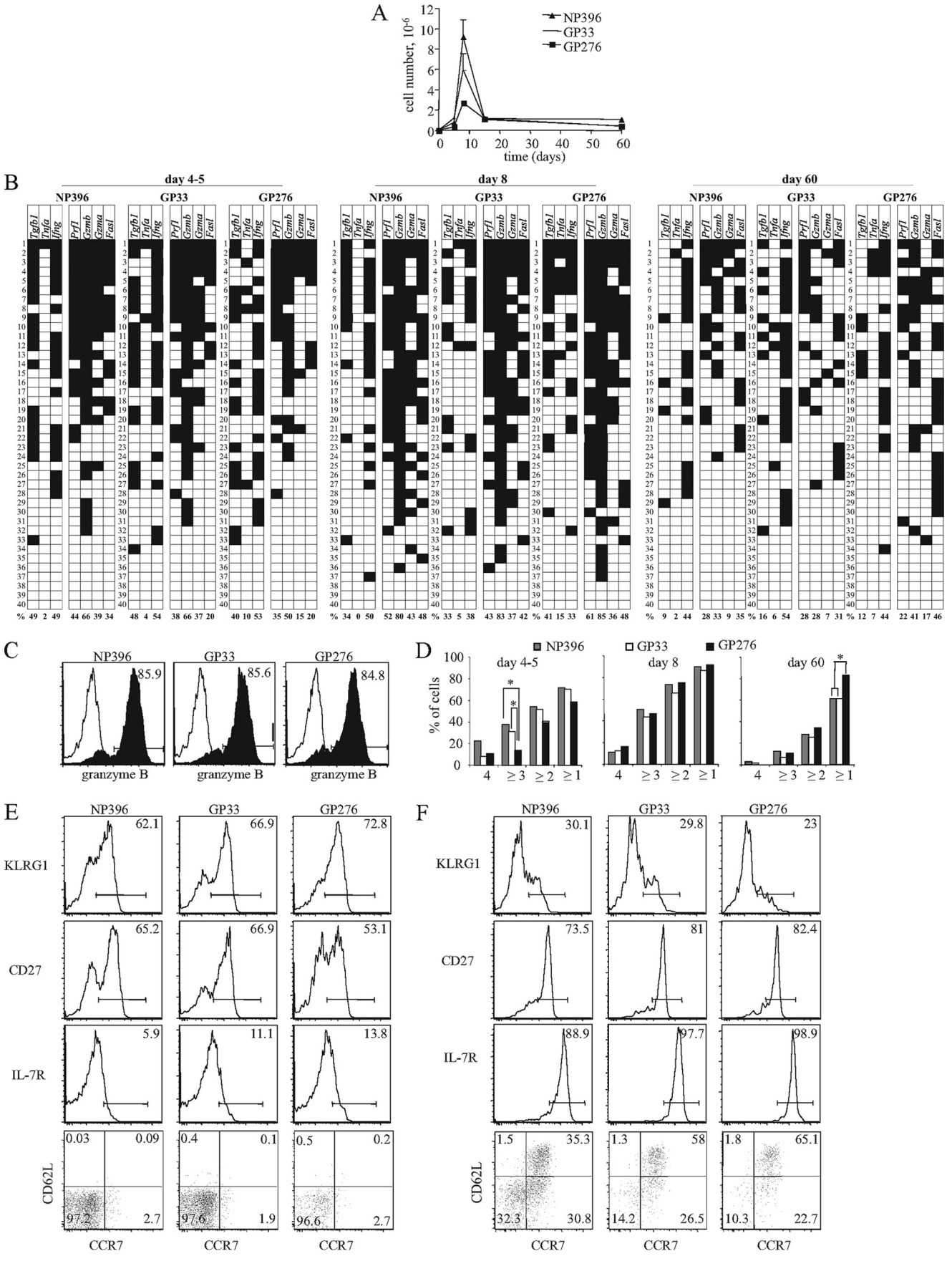
RESULTS

Endogenous CD8$^+$ T cells with different epitope specificities exhibit similar differentiation patterns after LCMV infection. Although a reproducible response hierarchy is found during LCMV infection in mice (15, 29), it still is unclear whether dominant and subdominant T cells are functionally distinct and/or whether their differentiation kinetics differ. To address this, first we screened responding cells for the expression of 14 effector genes known to be expressed by T lymphocytes (32). We found that during LCMV infection, only eight of these genes were expressed. We evaluated the pattern of these genes' expression in CD8$^+$ T-cell populations specific for two dominant LCMV epitopes (NP396 and GP33) and one subdominant epitope (GP276) following the infection of mice with LCMV Armstrong, which should cover all effector mediators during this response. The ratio between the most and the least abundant populations (NP396 and GP276 specific) was approximately 1:3 to 1:4, and the size of the GP33-specific population was between these levels (Fig. 1A), which is consistent with observations made in previous studies (29). At days 4 to 5 postinfection, ex vivo cytokine gene expression was identical between cells responding to all three epitopes, and approximately half of screened cells expressed *Ifng* and *Tgfb1* (although they were not always coexpressed), while *Tnf* was expressed in only a minority of cells (Fig. 1B). The frequency of cells expressing effector molecules with direct cytotoxic potential (*Gzma*, *Gzmb*, and *Prf1*) was indistinguishable between GP33- and NP396-specific populations. The only statistically significant difference between the two dominant and the subdominant population was that the latter expressed less *Gzma* (Fisher's exact test; $P = 0.0236$ for NP396 versus GP276 and $P = 0.0172$ for GP33 versus GP276). However, at day 8 the differences were abolished and all populations expressed the individual genes with a similar frequency (Fig. 1B). At the same time, all of the cytotoxic effector genes (*Prf1*, *Fasl*, *Gzma*, and *Gzmb*) reached the peak of their expression. Due to a lack of suitably optimized antibodies to evaluate native perforin, we could quantitate protein levels of only granzyme B at the single-cell level. Intracellular staining for granzyme B demonstrated that mRNAs were actively translated into large amounts of protein, as the percentages of cells expressing granzyme B mRNA and protein were approximately equal (Fig. 1C). To simplify the analysis of gene coexpression (and to provide an estimate of the cytotoxic potential of the cells in each population), we determined the number of cells that coexpressed cytotoxic genes (*Prf1*, *Gzmb*, *Gzma*, and *Fasl*) and calculated the cumulative proportion of cells expressing all four of these mRNAs, ≥ 3, ≥ 2, or ≥ 1 (Fig. 1D). On day 8 postinfection, approximately 90% of all cells expressed at least one of these cytotoxic effector genes, while 40 to 50% coexpressed three or more.

We have shown previously that during maturation from the effector to the memory phase, OT-1 and HY TCR Tg cells markedly reduced effector gene expression and coexpression (32). These findings were confirmed in all three endogenous populations analyzed here. At day 60, the frequency of the expression of the majority of effector genes was much lower than that at the peak of cell expansion, and the cells showed a low level of gene coexpression (less than 30% of cells expressed two or more genes together) (Fig. 1B and D). While a similar proportion of NP396- and GP33-specific cells expressed at least one of the cytotoxic effector genes, a slightly higher proportion of GP276-specific did so (Fig. 1D). Importantly, however, the percentage of cells expressing each individual gene was not significantly different in memory populations of different immunodominance. Overall, these results show that dominant and subdominant populations do not show major differences in effector gene expression patterns. Besides, cytokine expression after T-cell activation previously failed to discriminate between differences of cell populations recognizing different LCMV peptides (29).

We next determined if differences in immunodominance have an impact on the expression of cell surface markers associated with CD8 differentiation. At the peak of the response, LCMV-specific T cells had fully downregulated CCR7, CD62L, and IL-7R and upregulated CD27 and KLRG1 (Fig. 1E). While dominant NP396- and GP33-specific cells scored similarly for all of these parameters, GP276-specific cells showed a slight decrease in CD27 expression and an increase in KLRG1 expression. This difference could be due to the delayed kinetics of GP276-specific cells we already detected in our gene expression analysis and that disappeared in the memory phase. As described previously, LCMV-specific memory cells reexpressed CCR7, CD62L, and IL-7R, further upregulated CD27, and downregulated KLRG1 (Fig. 1F) (41, 50). Since, in contrast to what is found in human T cells, CCR7 and CD62L are not necessarily coexpressed in mouse memory cells (42), we evaluated the coexpression of these two markers. Indeed, we found a significant fraction of CCR7$^+$ CD62L$^{low/-}$ cells in all LCMV-specific memory populations, i.e., these cells could not be classified as either T-cell central memory (T_{CM}) or T-cell effector memory (T_{EM}) (Fig. 1F). Besides, both CCR7$^-$ and KLRG1$^+$ cells expressed IL-7R, in contrast to what is found in human cells. Therefore, the overall phenotype of LCMV-specific mouse memory cells contrasts to that found in human populations, where T_{CM} CCR7$^+$ cells express CD62L and the loss of CCR7 and KLRG1 correlates with IL-7R downregulation. It also must be noted that human T_{CM} and T_{EM} populations have very different functional properties and gene expression profiles even when studied using our single-cell strategy (25, 28), while the gene expression of the NP396-specific cell cohort, which had more CCR7$^-$ and CD62L^{low} cells, was similar to that found in other cell types (Fig. 1B). These results indicate that T_{CM} and T_{EM} human memory populations have no direct equivalent in the mouse.

The comparison of memory cell phenotypes showed variations both between individual mice studied in the same experiment and between experiments (see below). However, the NP396-specific cohort frequently had higher frequencies of CCR7$^-$ CD62L$^-$ cells and a slight reduction of CD27^{high} representation than cells with other LCMV specificities, but other phenotypes were equivalent. These results confirm that cells recognizing the NP396 epitope have a slower kinetics of CD62L upregulation (35). However, the reduced expression of CD62L in the dominant NP396-specific population contradicts the notion that more abundant clones preferentially upregulate CD62L (30).



Long-term gene expression at the memory stage is not due to viral latency. As $CD8^+$ T-cell differentiation during LCMV Armstrong infection resulted in the generation of a substantial fraction of memory cells expressing mRNAs for at least one cytotoxic effector component, we considered the possibility that viral clearance is incomplete, and that although it is undetectable by plaque-forming assays, virus may be persisting at low levels, as has been found to occur with more pathogenic LCMV strains such as LCMV WE (7). CD8-deficient or CD8-depleted mice are unable to contain the virus (11), so we reasoned that if virus was latent, it would reappear if T cells were depleted. We thus rendered a group of mice that had resolved an acute LCMV infection immunodeficient by depleting them of T cells and tested them for the reemergence of virus. Plaque-forming assays carried out on lymphoid tissues and various peripheral organs (brain, kidney, testis, liver, and lung) of T-cell-depleted animals were all negative (data not shown), strongly arguing against the possibility that chronic low-level stimulation by LCMV was provoking long-term gene expression.

We have noted previously that the quantity of mRNA expression for each memory CD8 T cell was significantly below the levels found at the peak of the response (32). Moreover, memory cells did not express measurable levels of granzyme B protein (Fig. 2A) and did not secrete IFN-γ without restimulation (data not shown), confirming previous findings (51). Nevertheless, in contrast to naïve cells, they were capable of performing peptide-pulsed target elimination after 12 h, which was remarkably similar to target elimination by effector cells (Fig. 2B). This analysis confirmed our previous findings that mRNA profiles constitute sensitive means of predicting the cytotoxic potential of CD8 cells (32).

Monoclonal TCR Tg cells and the endogenous cells of the same specificity have identical differentiation patterns. As we did not observe any striking differences in the expression profiles of several cytokine and cytotoxic effector molecules between the dominant and subdominant populations, we asked if a more robust immunodominance hierarchy established upon the adoptive transfer of high numbers of TCR Tg cells, in which the latter would dominate the endogenous repertoire, would reveal the differences in effector and memory cell

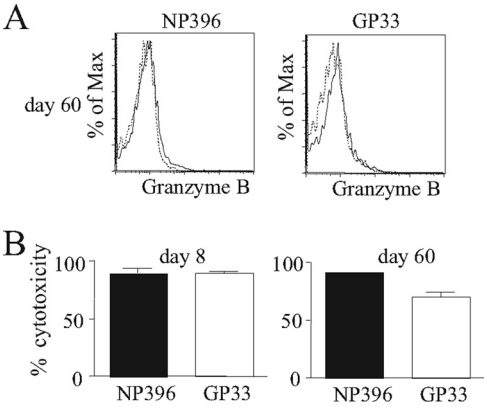


FIG. 2. Long-term gene expression in memory cells is not due to viral latency. B6 mice were infected with 2×10^5 PFU of LCMV Armstrong. (A) Intracellular granzyme B expression was analyzed on days 8 and 60 postinfection for the indicated epitope-restricted populations (black line). The isotype control staining of the same cell populations also is shown (dashed line). The profile shown is representative of results obtained from three individual mice. (B) An in vivo cytotoxicity assay also was performed on days 8 and 60 postinfection, and specific cytotoxicity is depicted; the results shown are the mean of data from three individual mice, and the error bars indicate standard errors of the means.

generation. We initially compared the functional properties of LCMV-specific memory cells generated after the adoptive transfer of large (5×10^5) and small (5×10^3) numbers of P14 cells. Given an estimated engraftment level of 10% (5), the low-dose adoptive transfer likely would have resulted in a precursor frequency approaching that of the endogenous GP33-specific precursors, while the high dose likely exceeded it by more than 100-fold (5, 24, 30). Importantly, memory P14 cells originating from large and small cell numbers had similar IFN-γ and TNF-α secretion potential upon in vitro restimulation (Fig. 3A), arguing against the previously suggested idea that a shifted $CD8^+$ T-cell maturation at the beginning of the response has a long-term effect on memory T-cell functions (2).

We next compared the early phases of the response. The

FIG. 1. Differentiation patterns of CD8 cells recognizing dominant and subdominant LCMV epitopes show marked similarity. B6 mice were infected with 2×10^5 PFU of LCMV Armstrong. (A) The number of NP396-tet^+, GP33-tet^+, and GP276-tet^+ cells in the spleen was analyzed at different time points after infection. The results shown are the means of values from three to six mice tested in two separate experiments, and the error bars indicate one standard error above the means (SEM). (B) Individual cells of each epitope specificity were recovered at the indicated times (days) postinfection from six individual mice in two independent experiments and were tested directly ex vivo for the coexpression of the indicated effector mRNAs. Forty-five to 90 cells of each specificity were evaluated per time point. Only wells that were positive for *CD3*ε (indicating that they contained a cell) were included in the analysis. Since we did not find significant variation between mice and between experiments, the data were pooled. Each horizontal row represents the pattern of gene expression in the same single cell; representative results from 40 cells are shown. Gene expression is indicated in black, and negative results are shown in white. Cells are ordered by the number of cytotoxic effector genes they expressed. The percentages at the bottom of each column represent the frequency at which the indicated gene was expressed in the whole population analyzed. (C) On day 8 postinfection, NP396-, GP33-, and GP276-reactive cells were identified by tetramer staining, and granzyme B expression in each population was analyzed directly ex vivo by intracellular staining. The filled histograms represent granzyme B staining, and the white histograms show the staining of the same populations with an isotype-matched control antibody. (D) The number of mRNAs for cytotoxic effector genes (*Prf1*, *Gzma*, *Gzmb*, and *FasL*) coexpressed by each cell was calculated (0 to 4). The results are expressed cumulatively as the percentage of cells specific for a given epitope that coexpressed mRNAs for ≥1, ≥2, ≥3, or 4 of these genes at the indicated times postinfection (a cell expressing two genes would be included in both the ≥2 and ≥1 categories. Statistically significant differences (as determined using Fisher's exact test) are marked (∗, $P < 0.05$). (E) Phenotypes of LCMV- specific T cells at day 8 after infection. Graphs are from one individual mouse out of six mice studied in two independent experiments showing overlapping results. (F) Phenotypes of LCMV-specific memory cells. Results are from one mouse out of seven studied in three independent experiments. We found considerable variation in the expression of CD62L.

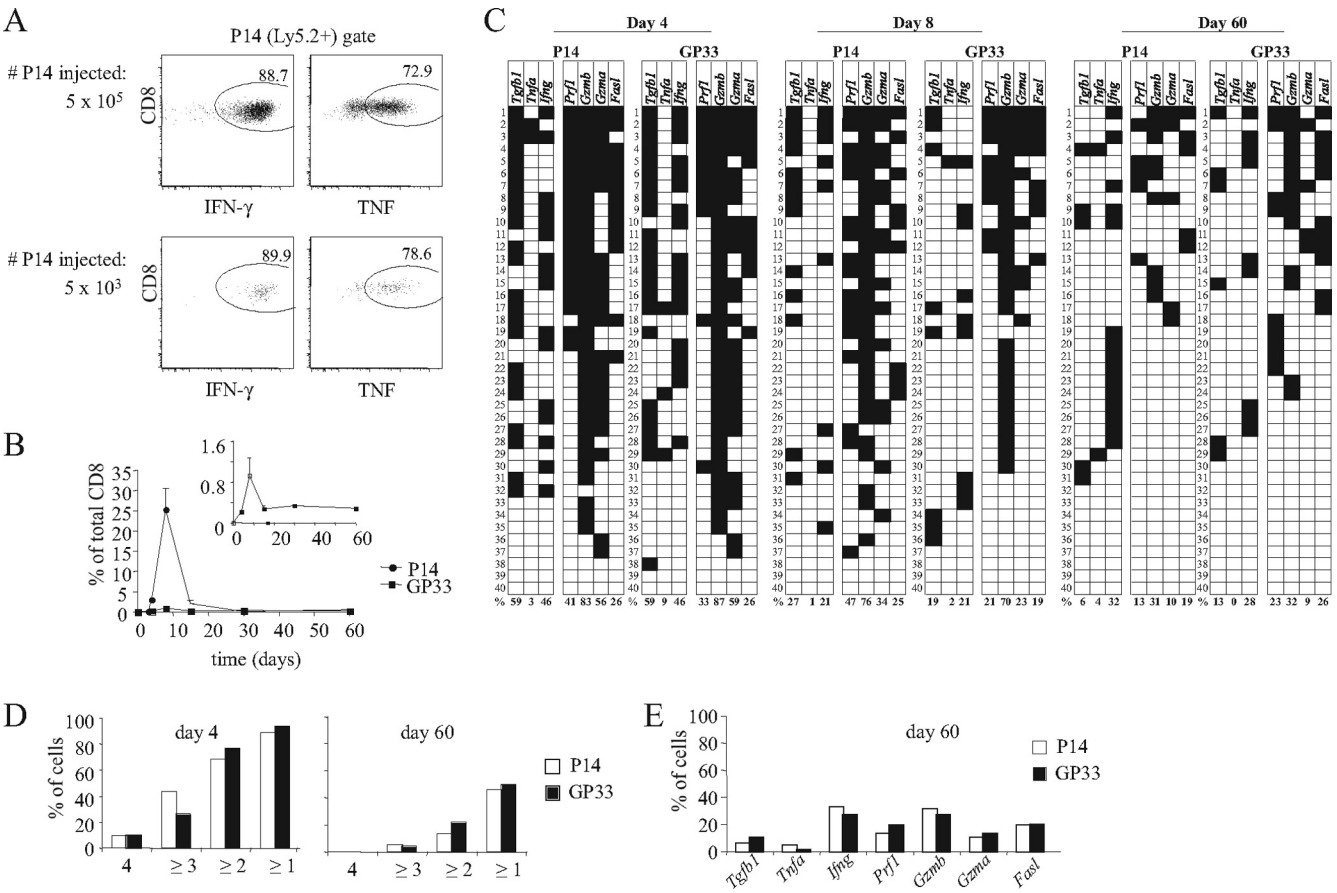


FIG. 3. Differentiation patterns of adoptively transferred TCR Tg cells and endogenous cells recognizing the same epitope in the same mice. (A) B6.Ly5.1 mice were injected with 5×10^5 or 5×10^3 P14 Tg cells (Ly5.2$^+$) and infected with 2×10^5 PFU of LCMV Armstrong. At day 90 postinfection, splenocytes were removed and restimulated in vitro with the GP33 peptide, and the proportion of P14 cells producing IFN-γ and TNF-α was analyzed by intracellular cytokine staining. The dot plots show representative results from one animal in each group and are gated on P14$^+$ cells. The percentage of P14$^+$ cells secreting each cytokine is indicated within the dot plots. (B to E) B6.Ly5.1 mice were injected with 5×10^5 P14 Tg cells (Ly5.2$^+$) and infected with 2×10^5 PFU of LCMV Armstrong. (B) The proportion of P14 and endogenous GP33-specific cells in the spleen was analyzed over time in the same mice. The mean results from three individual mice are shown, and the error bars indicate standard errors of the means. The inset shows a magnification of the endogenous cell graph. (C) Gene expression in individually sorted P14 and endogenous GP33-specific cells was analyzed; the results are presented as described for Fig. 1B. (D) The pattern of the coexpression of cytotoxic effector mRNAs also was analyzed and is presented as described for Fig. 1D. (E) The percentage of cells on day 60 expressing each of the indicated genes (cytotoxic effector genes as shown in Fig. 3B) was analyzed and is shown for 90 individually sorted P14 and 70 endogenous GP33-specific cells. The cells were obtained from three individual mice.

differentiation of CD8$^+$ T cells initially present at either high or low precursor numbers was analyzed previously in the different animals. However, adoptive transfers of $>10^5$ TCR Tg cells accelerate the speed of viral clearance (10, 56). It therefore was possible that the observed differences in T-cell differentiation kinetics (2, 26, 45) were attributable to the differences in the antigen clearance between mice left uninjected or injected with high frequencies of naïve cells. We therefore restricted our investigation to the comparison of P14 cells transferred at high precursor frequencies to endogenous cells present in the same mice to allow both populations to have an equal exposure to the infectious environment. The gene expression analysis of naïve P14 cells showed that rare cells expressed either *Tgfb1* or *Prf1* but none of the other effector genes (data not shown). At days 4 to 8 of the response, TCR Tg cells outnumbered endogenous GP33-specific cells by up to 30-fold (Fig. 3B), but the cytokine and cytotoxic effector genes transcribed in both populations were similarly represented. The resemblance between those populations also was apparent on the analysis of cytotoxic gene coexpression (Fig. 3D). Most importantly, memory TCR Tg and endogenous GP33-specific cells had the same expression frequencies for all screened genes (Fig. 3C and E). In summary, once putative differences in antigen loads are avoided, both effector and memory GP33-specific cells developing in the same mice from precursors initially present at widely disparate numbers had equivalent expression profiles for all genes tested.

It also was reported that high-dose naïve TCR Tg cell transfers induced the precocious upregulation of CD62L and IL-7R compared to that of equivalent populations injected at a low frequency (2). To determine if these differences also were due to accelerated response kinetics due to the faster resolution of the infectious stimuli, we compared the expression of these markers in normal mice and in P14-transferred mice infected

simultaneously with LCMV. One week after infection, the endogenous populations in normal mice had fully downregulated IL-7R and CD62L expression, while P14 Tg cells expressed higher levels of CD62L and IL-7R (Fig. 4A). However, this upregulation was not peculiar to populations present in high frequencies. In P14-injected mice, all LCMV-specific populations (either Tg or endogenous) also upregulated these markers. The abundant P14 and the rare GP33-specific endogenous populations of P14-injected mice expressed similar levels of IL-7R. Endogenous NP396- and GP276-specific cells also upregulated IL-7R expression, although they did so at slightly lower levels than those found in GP33-specific T cells. The CD62L expression was upregulated to equivalent levels in both Tg and endogenous cells of all peptide specificities. These results indicate that the precocious upregulation of these markers described after high-frequency adoptive transfers is not a property of dominant clones. Rather, it appears to be the consequence of accelerated response kinetics that are known to occur in these circumstances, since it affects all LCMV-specific populations present in the same mouse. In addition, we also failed to confirm that Tg memory cells expressed a predominantly CD62L^{hi} phenotype (Fig. 4B). Rather, we found a significant variation in different mice studied in the same experiment and between experiments.

Finally, we evaluated the impact of high-dose P14 adoptive transfers on endogenous response. As reported previously (5) and shown in Fig. 1, the absolute number of endogenous GP33-specific cells was reduced in P14-injected mice compared to that found in normal mice infected simultaneously (Fig. 4C). Surprisingly, P14 adoptive transfers did not substantially affect the T-cell responses to other LCMV epitopes (Fig. 4C and D). At both the response peak and at the memory phase, the number of NP396- and GP276-specific cells determined either by tetramer staining or their capacity to secrete cytokines after in vitro stimulation (Fig. 4D) was similar in P14-injected and normal mice. We conclude that the injection of P14 cells accelerates response kinetics, as shown by the modifications of IL-7R and CD62L in all cells responding to LCMV in P14 injected mice. Otherwise, it does not affect the properties of LCMV-specific cells or influence the endogenous responses to other LCMV epitopes.

TCR downregulation at the early stages of the response masks the detection of Ag-specific cells. A potential drawback to the use of tetramer staining to identify epitope-specific T cells for functional profiling is the TCR downregulation that follows T-cell activation. We tested whether tetramer staining is a reliable marker of Ag-specific cells during the immune response. The Tg cells that can be identified by an allotype marker showed a substantial loss of surface tetramer labeling during the expansion period. This effect was not immediate, sparing the first 2 days of the response, when TCR-Tg cells were activated but most had not divided (not shown). At day 3 of the LCMV response, we saw considerable mouse-to-mouse variability, with 30 to 70% of P14 cells being undetectable by tetramer labeling. By day 5, less mouse-to-mouse variation was observed, yet 25% of P14 cells did not label with tetramers (Fig. 5A). The comparison of tet^{neg} and highly tetramer-positive (tet^{hi}) populations showed that the former did not express CD3, confirming that the loss of tetramer binding was caused by TCR downregulation (Fig. 5B, upper row). We further tested if TCR downregulation could bias the evaluation of the properties of antigen-specific cells during the response. Indeed, tet^{neg} and tet^{hi} cells on day 3 of infection differed in CD69, CD27, CD25, and Ly6C expression levels (Fig. 5B, lower row), while IL-7R, CD122, and CD44 were expressed similarly (data not shown). To investigate if such downregulation was just an artifact induced by high-frequency transfers or could occur when antigen-specific naïve cells were present at a physiologic number, we studied Tg cells injected at low frequency. As expected, the kinetics of the Tg cell response was much slower. At day 4, TCR downmodulation was evident but Tg cells were very rare. We found a considerable TCR downmodulation even at day 5 of the response, when more than half of the Tg pool was failing to bind tetramers (Fig. 5C).

We aimed to investigate if TCR downregulation also could bias the evaluation of the normal endogenous response. Since tet^{neg} endogenous cells cannot be visualized, we compared tet^{hi} and tetramer-intermediate (tet^{int}) cells in normal mice (Fig. 5D). Importantly, tet^{int} cells had substantially higher granzyme B expression than tet^{hi} cells (Fig. 5D), directly linking the activation status measured by TCR downmodulation to a different effector profile. Thus, although we lack the means to test for the endogenous cells that are completely tet^{neg} (since these cells do not express TCR they also should not score as IFN-γ producers after in vitro stimulation), our results strongly suggest that our current methods of detection fail to identify a substantial fraction of antigen-specific cells during the expansion phase, and moreover, they introduce bias in the evaluation of the properties of antigen-specific cells from normal mice.

Cytokine and cytotoxic effector gene expression are not synchronized. As T cells do not reach lymphoid organs synchronously and are exposed to highly varied microenvironmental stimuli, cells at various differentiation stages are found at any given time. Having established that TCR Tg cells allow us to fully assess the early dynamics of the CD8 differentiation, we further subdivided their progression steps by a combination of CFSE and CD69 labeling. CFSE labeling allowed us to focus on the majority of P14 cells (>95%) that have divided four or more times by day 3 (Fig. 6A). The CFSE-low P14 population was further subdivided into more recently and less recently activated subsets on the basis of the differential expression of an early and transient T-cell activation marker, CD69 (34); less advanced CD69$^+$ and more advanced CD69$^-$ cells were sorted. When gene expression patterns were analyzed, cytokine gene expression was found to differ from the expression of cytotoxic effector genes, and in general, cytotoxic effector genes were transcribed longer than cytokine genes (Fig. 6B). Cytokine expression consistently peaked early (on day 3 or before), there was no difference between CD69$^+$ and CD69$^-$ cells (Fig. 6B), and a highly significant drop of expression occurred during the next day (Fig. 6C). On the other hand, the transcription of cytotoxic effector genes varied: *Gzmb* and *Prf1* peaked early, while *Gzma* and *Fasl* were present only in a minority of CD69$^+$ cells, and a significant rise in their expression occurred as they progressed to the CD69$^-$ stage (for *Gzma*, $P = 0.0001$; for *Fasl*, $P = 0.002$). Notably, the generation of *Il2* mRNA was found rarely in ex vivo P14 cells (less than 5% of total cells; data not shown) at any of the stages tested (days 3, 4, 8, 15, 30, and 60). The latter finding casts doubt on the physiological significance of reports that TCR Tg

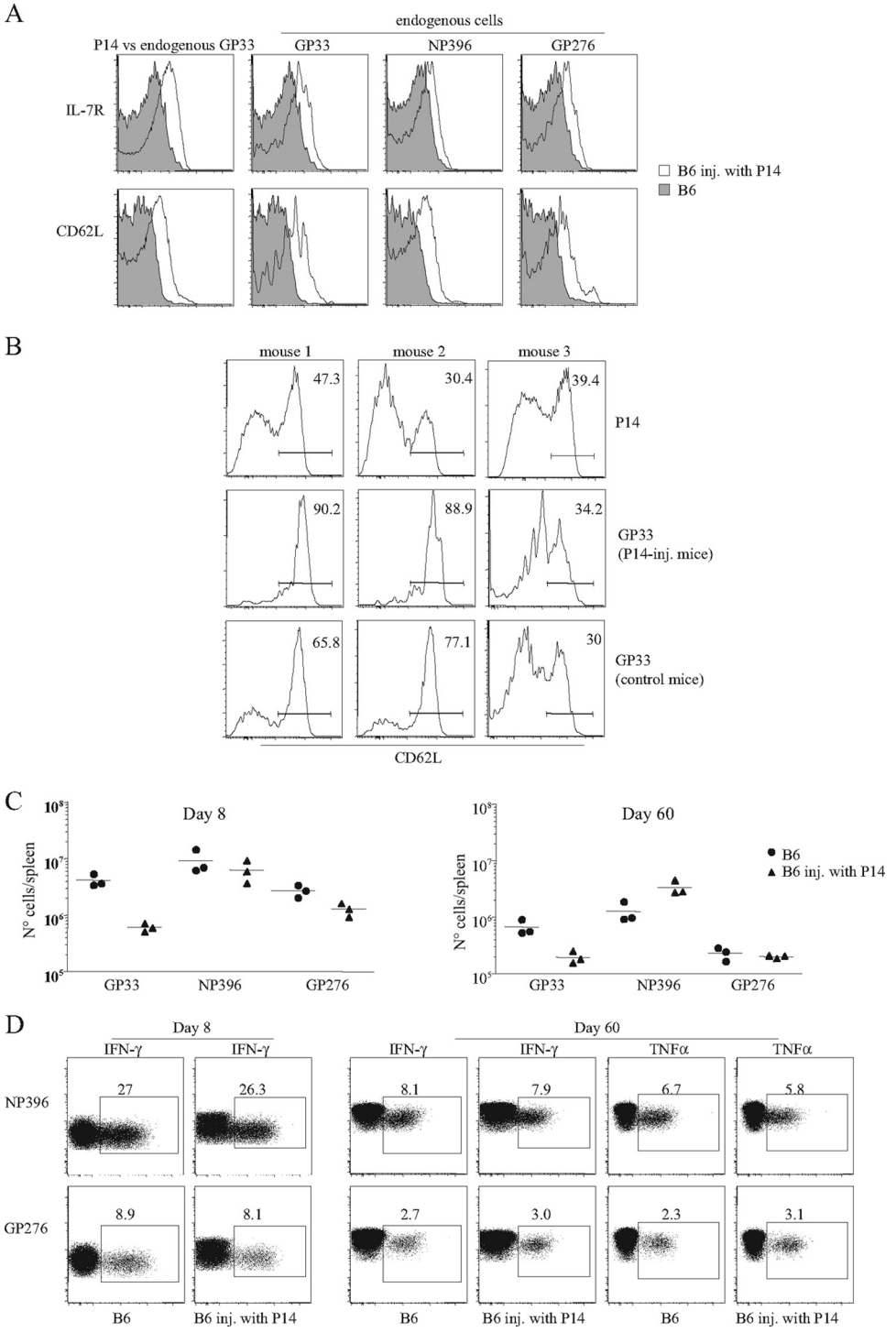


FIG. 4. Impact of high-dose naïve Tg transfers on the endogenous response. B6.Ly5.1 mice left untreated or were injected with 5×10^5 P14 Tg cells (Ly5.2$^+$) and were infected simultaneously with 2×10^5 PFU of LCMV Armstrong and studied at days 8 and 60 after infection. (A) CD62L and IL-7R expression in cells of with different peptide specificities at day 8 after infection. Histograms compare CD62L and IL-7R expression levels of CD8 cells with the indicated peptide specificities in 1 P14 injected (inj.) (open graphs) and 1 noninjected B6 mouse (gray) of 12 mice studied in two independent experiments. On the far left, P14 cells (open histogram) are compared to GP33-specific noninjected mice. (B) Variation of CD62L expression in GP33-specific cells 2 months after infection. Graphs compare Tg cells (upper) to endogenous cells present in the same mouse (middle). The lower graphs show endogenous cells in the mice that were not injected with P14 cells. (C) Absolute numbers of cells of different peptide specificity at day 8 (left) and 2 months (right) after infection. Results show individual mice from one experiment out of two with equivalent results. (D) IFN-γ expression after in vitro stimulation with NP396 and GP276 peptides at day 8 (left) and 2 months (right) after infection.

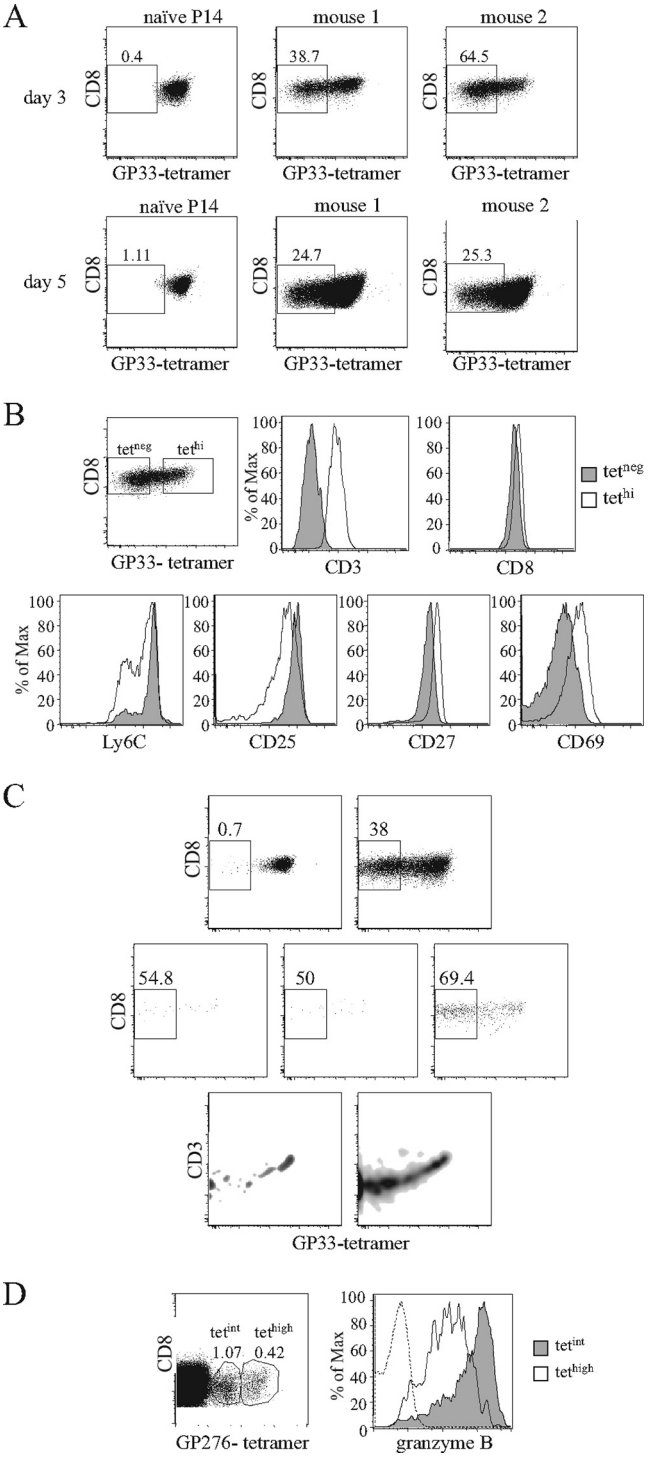


FIG. 5. MHC-I tetramer labeling during the expansion phase. B6.Ly5.1 mice left untreated or receiving Ly5.2$^+$ P14 Tg cells were infected with 2×10^5 PFU of LCMV Armstrong and studied at different time points after infection. (A and B) Mice were injected with 5×10^5 Tg cells. (A) Results compare GP33 tetramer (tet) binding in Ly5.2$^+$ P14 naïve cells and in P14 cells at on day 3 (top) and day 5 (bottom) after infection. Staining is from individual mice representative of four experiments with two to three mice per time point. (B) On day three after infection, P14 cells were arbitrarily subdivided into tet^{neg} (gray) and tet^{hi} (white) subsets, and each population was tested for the expression of the indicated cell surface molecules. Gates for tet^{neg} cells were established in noninfected B6 mice and for tet^{hi} in naïve Tg cells. (C) B6 mice were injected with either 5×10^5 or 10^4 Ly5.2$^+$ P14 Tg cells and studied at days 4 and 5 after infection. Results are for GP33 tetramer binding in P14 Tg cells. Naïve mice (upper left), cells from mice injected with 5×10^5 naïve cells (upper right), and cells from three individual mice injected with 10^4 naïve cells (lower graphs) are shown. At day 4, very few Tg cells were detected in the latter mice. (D) Granzyme B expression in cells expressing different tet binding intensity in normal mice. CD8 cells were recovered 5 days after infection, labeled with GP276 tetramers, and subdivided into tet^{int} and tet^{hi} populations. Results show the gates used for such subdivision and intracellular granzyme B staining for tet^{int} (gray) and tet^{hi} (white) populations; the dashed lines represent the staining of the same cells with an isotype control antibody.

effector CD8 cells arising from high precursor frequencies are more likely to secrete IL-2 upon in vitro stimulation (2). *Il7r* downregulation (Fig. 6C) followed the kinetics previously described on a protein level (19). Furthermore, we observed that *Ccr7* expression rapidly dropped between days 3 and 4, most likely participating in the release of more mature Ccr7$^-$ cells from T-cell-restricted areas of secondary lymphoid tissues. As we have demonstrated that gene expression in LCMV-specific cells evolves very fast over short periods of time and at defined differentiation milestones (such as the CD69$^+$→CD69$^-$ transition), the analysis of other markers of cell progression/fath (17) is expected to provide us with an even richer picture of gene differentiation dynamics and cell heterogeneity.

DISCUSSION

TCR Tg cells commonly are used in the assessment of the properties of T cells. They are easy to manipulate and to visualize. Their defined TCR expression allows one to monitor the same clone throughout the immune response (39, 40). This characteristic is fundamental to determine if the changes in population properties throughout the response are due to the selection of particular clones of antigen-specific cells. However, it was suggested recently that when TCR Tg cells are present at high precursor frequencies, their intraclonal competition for antigen leads to their suboptimal activation and abnormal differentiation (2, 26, 45). Several reasons prompted us to reexamine this claim in greater detail. First, several studies showed that a short-term contact with an antigen is sufficient to trigger a complete CD8 differentiation program (18, 46), and that extensive CD8 expansion is not a prerequisite for efficient memory generation (3). Second, in other studies effector CD8 numbers seem to hit a similar ceiling regardless of initial variability in precursor numbers or specificity (22), arguing for stimulation-tailored rather than T-cell-intrinsic differentiation pathways. Finally, other data suggested alternative explanations to the different behavior of high- and low-density TCR Tg cell transfers. The adoptive transfers of $>10^5$ TCR Tg cells have been shown to alter the kinetics of pathogen clearance and the timing of peak CD8$^+$ T-cell expansion (10, 35, 56). Since high- and low-dose transferred populations were studied systematically in different recipients where antigen loads and antigen clearance are known to be different, alterations in the course of infection could account for the different population properties in mice that received different numbers of TCR Tg cells.

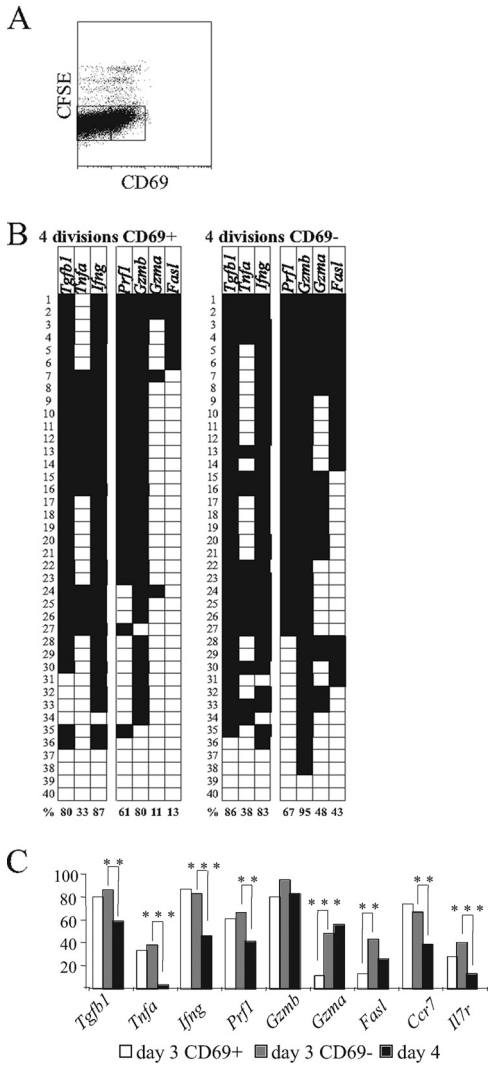


FIG. 6. Rapid progress of $CD8^+$ T-cell differentiation during early infection can be monitored using a combination of TCR Tg cells, CFSE division profiles, and CD69 expression. B6.Ly5.1 mice were injected with 5×10^5 P14 Tg cells ($Ly5.2^+$) and infected with 2×10^5 PFU of LCMV Armstrong. (A) At day 3 postinfection, splenocytes were stained with CD69, and division profiles were analyzed by the evaluation of CFSE expression. Cells that had divided four or more times were sorted into two subpopulations ($CD69^+$ and $CD69^-$) based on the indicated gates. (B) Gene expression in individually sorted $CD69^+$ and $CD69^-$ P14 cells was analyzed on day 3 postinfection; the results are presented in the same format as that used for Fig. 1B. (C) The progression of gene expression in individually sorted P14 cells was analyzed as the linear maturation of cells occurred (i.e., in the following sequence: day 3 $CD69^+$ cells [white]→ day 3 $CD69^-$ cells [grey]→ day 4 [black]). Statistically significant differences between progressive differentiation stages are marked (** and ***, $P < 0.01$ and $P < 0.001$, respectively.

Competition for antigen and clonal competition also occur in normal immune responses and contribute to the immunodominance hierarchy observed. A partial or complete compensation for a loss of a particular epitope by other specificities has been known to occur (1, 21, 24, 36, 44). In some circumstances it has been suggested that the cytokine-mediated active suppression of dominant clones over subdominant ones (immunodomination) occurs (48, 52), but the existence of such active immunosuppression still is disputed (24). Besides, although immunodominance has been studied widely in many infectious models, it still is unclear whether dominant and subdominant populations diverge in their functional capacities and protection capabilities (6, 20, 48). To address these issues, in addition to conventional tests, we performed a powerful single-cell multigene expression study of several antigen-specific populations during the course of LCMV infection in mice. When studied in the same infectious context in the same mice at the same time point of the response, the T-cell populations of different specificities and present at different frequencies showed remarkably similar features. Thus, except at the earliest stages of infection (days 4 to 5) when GP276-specific cells expressed less granzyme A mRNA than NP396- and GP33-specific cells, dominant and subdominant cell effector and memory had remarkably similar cytokine (*Ifng*, *Tnf*, and *Tgfb1*) and cytotoxic gene expression (*Prf1*, *Gzmb*, *Gzma*, and *Fasl*) and coexpression profiles. Previous comparisons of cytokine profiles after in vitro stimulation also failed to reveal major differences (49), and we found that cell surface markers' expression most frequently was overlapping. As an exception, the subdominant GP276-specific population showed some delay in CD27 upregulation and KLRG1 downregulation at day 8, but these differences disappeared in the memory phase, when these cells' phenotypes were equivalent to those found in GP33-specific cells. Conversely, the NP396-specific memory cohort usually had a larger fraction of $CCR7^-$ $CD62L^{low}$ cells than cell populations with other peptide specificities, but otherwise they expressed the same KLRG1 and IL-7R labeling, and it was reported previously that this cell type eventually also upregulates the expression of both of these ligands. Overall, these data directly argue against the hypothesis that dominant and subdominant populations follow disparate differentiation pathways. These findings were confirmed even when major differences in clonal abundance were introduced artificially by the adoptive transfer of Tg cells.

The differentiation profiles of monoclonal T-cell populations recently have fallen under scrutiny, since several reports suggested that the artificial introduction of TCR Tg CD8 cells in numbers exceeding those of endogenous cells of similar epitope specificity (5, 24, 30) resulted in the inadequate differentiation of TCR Tg cells (2, 26, 45). These reports, however, focused mainly on CD62L and IL-7R expression analysis, and functional assays were performed only at a single time point of the infection. These studies also did not take into consideration possible differences in response kinetics that could result from the introduction of a large cohort of naïve Tg cells. Indeed, abundant and rare clone behavior always was studied in different mice, where Tg cells could be submitted to different antigen loads and abundant and rare clone accumulation peaked at different time points (2). Supporting the notion that previously reported differences between high- and low-dose transfers can be explained by a different response kinetics, adoptive transfers of $>10^5$ precursors were shown to accelerate the kinetics of pathogen clearance and CD8 expansion (10, 35, 56).

Contrary to those studies, we compared TCR Tg and endogenous cells of the same epitope specificity from the same animals, where both faced exactly the same antigen exposure

and showed similar response kinetics. Moreover, when Tg cells are present, the endogenous GP33-specific population expands very little, which should prevent any TCR downregulation early in the response. We found that under these conditions, Tg and endogenous GP33-specific CD8 cells retrieved from the same mice always were remarkably similar. They not only had initiated IL-7R and CD62L upregulation precociously at day 8 but also showed similar phenotypes and gene expression profiles at the response peak. The analysis of CD8 T cells with other specificities in these transferred mice also supported the notion that high-dose transfers only accelerate response kinetics. Indeed, we found that in P14-injected mice both NP396- and GP276-specific populations also had initiated IL-7R and CD62L upregulation at day 8 after infection. Surprisingly, these cells appeared to be otherwise unaffected by the presence of high frequencies of TCR Tg cells. Their frequency and their capacity to produce IFN-γ was similar in mice left untreated or receiving P14 Tg cells. These results demonstrate that high-frequency adoptive transfers do not inhibit overall endogenous responses but only influence the expansion of T-cell populations with the same TCR specificity.

Our results also do not support the notion that high-frequency transfers induce major modifications in the properties of memory cells. We demonstrated that memory cells on day 90 that arose from 5×10^3 and 5×10^5 P14 cells did not differ in functional capacities such as stimulation-induced cytokine secretion. We did not find evidence for the predominant generation of $CD62L^+$ Tg memory cells in high-frequency transfers. In our hands, the GP33-specific endogenous memory cells frequently expressed more CD62L than the Tg memory cells. Differences between the present and previously published results (2, 26) could be due to mouse-to-mouse variability, as we found in our experiments, or to the fact that we always evaluated endogenous and Tg cells present in the same mouse. Our results suggest that studies showing a preferential expression of CD62L in high-frequency cells were not exhaustive, and that the conclusion that these cells only generate $CD62L^+$ T_{CM} (a major argument to suggest abnormal differentiation) is unreliable.

Overall, these data suggests that high-frequency adoptive transfers just accelerate response kinetics, and that Tg cells only compete with the endogenous cells that share the same TCR specificity. It is likely that such competition is greatly influenced by the relative avidity/cross-reactivity of the TCR Tg cells with respect to the average avidity/cross-reactivity of the endogenous antigen-specific cells. Different Tg CD8s populations were classified according to these parameters in the hierarchy OT1 > P14 > anti-HY (13), which appears to correlate directly with their inhibitory effect on endogenous responses. Indeed, the transfer of the high-avidity/cross-reactive OT-1 clone virtually abrogates endogenous responses, while P14 transfers have a smaller effect (2). In contrast, in high-frequency anti-HY Tg transfers to normal mice, the endogenous cells partially outcompete the Tg population. Both Tg and endogenous responses show reduced amplitude and become similarly represented in the overall anti-HY response (47).

TCR downregulation is a rapid and dose-dependent corollary of T-cell activation in vitro (43) but is rather transitory, lasting for about 24 h. TCR downregulation also was detected in acute infections in vivo (8, 54), but due to the lack of other markers to identify antigen-specific cells, these previous studies could not evaluate fully the extent of this phenomenon. Here, we established that Tg cells identified by an allogeneic marker, even when present at physiologic frequencies, downregulated TCR expression, and a major fraction fully lost TCR cell surface expression and failed to bind tetramers. This behavior is likely a common feature of CD8 immune responses, since we also found it in other infectious models and in other TCR Tg cells (P14 or OT-1 cells immunized with *Listeria*-expressing GP33 [LM-GP33] or LM-OVA, respectively; unpublished data).

Several aspects of this phenomenon must be emphasized. In contrast to the transient loss of TCR after in vitro activation, in vivo responding populations could remain TCR negative throughout a long time period during the expansion phase; activation status and tetramer binding were inversely correlated, allowing for the possibility that more activated cells could be rendered completely invisible by prominent TCR downregulation.

To summarize, the detailed analysis of CD8 T cells responding to different LCMV epitopes in the same infectious environment showed that relative clone abundance or TCR specificity did not alter substantially the properties of effector and memory cells. From this perspective, the current notion that high-frequency transfers of naïve Tg cells induce abnormal T-cell differentiation must be toned down. We found that differences in Tg behavior can be explained by a different response kinetics, that abundant Tg and rare endogenous cells with the same peptide specificity had overlapping properties, and that Tg cells did not affect the amplitude or the quality of the endogenous response to other LCMV peptides. It also was demonstrated recently that high-frequency transfers did not affect the quality of the memory responses (53). In contrast, the use of TCR Tg cells that can be recognized by allotype markers revealed that during acute infection, when high viral loads are present, a substantial fraction of responding cells downregulate their TCR and fail to bind MHC tetramers, and that tet^{pos} and tet^{neg} cells have different properties. Therefore, TCR-Tg mice may be fundamental for the evaluation of the entirety of the early immune response.

Finally, the important and long-lasting loss of TCR expression we found to occur during the expansion phase has major implications for our capacity to study early events in the vast majority of acute infections in the mouse (when Tg cells are not available) and, more importantly, in humans. Studies based on the tetramer binding identification and/or magnetic bead purification of antigen-specific cells likely are incomplete and biased (14, 30), since they select subpopulations with peculiar properties that do not represent the overall characteristics of the responding peptide-specific cohort. Moreover, it is at present unclear if any of the methods currently used to identify responding cells will be able to do so and in which circumstances. The failure to bind tetramers is due to TCR downregulation. It therefore is possible that the vast majority of tet^{neg} cells also are undetected through cytokine expression after in vitro stimulation with specific peptides, since these responses depend on the cell surface expression of the peptide-specific TCR. Moreover, we found that TCR downregulation increased when higher doses of virus were injected, suggesting

that the higher the viremia the more incomplete will be our assessment of the acute response. This important pitfall must be taken into consideration: we may fail to detect a major cohort of responding cells when high virus loads are present.

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RÉSUMÉ

Les cytokines de la famille γ_c sont essentielles au développement, à la différenciation thymique et à la survie périphérique des lymphocytes T naïfs. Transmettant leurs signaux par des récepteurs qui ont en commun la chaîne γ_c, les interleukines -2, -7, -15 et -21 sont des facteurs solubles pléiotropes. De par leur redondance lors d'une réponse immunitaire, le rôle individuel des cytokines γ_c dans l'homéostasie des lymphocytes T CD8 et dans la réponse anti-virale n'a été que partiellement élucidé. De plus, l'état actuel des connaissances ne permet pas de savoir avec précision à quel moment de la différenciation et selon quels mécanismes ces cytokines interviennent.

Afin d'évaluer le rôle des cytokines γ_c dans l'homéostasie des lymphocytes T CD8 naïfs, nous avons comparé des cellules monoclonales CD8 issues de souris TCR transgéniques P14 γ_c-compétentes ou γ_c-déficientes. Nous avons montré que les cellules T CD8 naïves $\gamma_c^{-/-}$ ne s'accumulent pas dans les organes lymphoïdes secondaires et que les quelques cellules résiduelles se caractérisent par une petite taille, une diminution de l'expression du CMH de classe I et une augmentation de l'apoptose. Nous avons ensuite corrigé le défaut intrinsèque de survie des cellules T CD8 $\gamma_c^{-/-}$naïves, en surexprimant la molécule humaine Bcl-2, un facteur anti-apoptotique. Cette approche nous a permis de restaurer le nombre de lymphocytes T CD8 naïfs en périphérie, malgré l'absence de chaîne γ_c. Par contre, tout comme ce qui avait été démontré pour les cellules T CD4, l'expression de Bcl-2 ne permet pas de corriger le défaut de taille et de synthèse protéique des cellules γ_c-déficientes. Nous concluons donc que les cytokines γ_c génèrent des signaux Bcl-2-dépendants et Bcl-2-indépendants pour maintenir le phénotype et l'homéostasie des lymphocytes T CD8 naïfs.

Afin de définir l'implication précise des cytokines γ_c au cours de la différenciation des cellules T CD8, nous avons évalué la réponse des cellules T CD8 Bcl-2^+ $\gamma_c^{+/+}$ ou $\gamma_c^{-/-}$ après infection par le virus de la chorioméningite lymphocytaire. De façon tout à fait étonnante, nous avons démontré que de nombreuses étapes de la réponse anti-virale primaire se déroulent normalement en l'absence de chaîne γ_c. En effet, l'expansion clonale, les changements phénotypiques associés à une activation et l'acquisition de fonctions effectrices par les lymphocytes T CD8 γ_c-déficients sont préservés. Par contre, les signaux dépendants de la chaîne γ_c s'avèrent essentiels à la différenciation et la prolifération des effecteurs tardifs ainsi qu'à la génération et le maintien des lymphocytes T CD8 mémoires. Nous proposons donc que les cytokines γ_c-dépendantes ne sont pas indispensables à l'acquisition de fonctions cytotoxiques et à la réponse anti-virale, mais génèrent des signaux Bcl-2-indépendants essentiels à la survie et à la prolifération des cellules T CD8 mémoires.