Neuroblastoma and gastrointestinal stromal tumor as a target for natural killer lymphocytes : the role of ncr3/nkp30

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NEUROBLASTOMA AND GASTROINTESTINAL STROMAL TUMOR AS A TARGET FOR NATURAL KILLER LYMPHOCYTES: THE ROLE OF NCR3/NKp30

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“Homo sum,
humani nihil a me alienum puto”

Terenzio, Heautontimorumenos v. 77

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ABSTRACT

Since Burnet and Thomas formulated in 1957 the cancer immunosurveillance theory, the scientific world has made tremendous progress to identify the immune cells involved in this process. Natural Killer (NK) cells have emerged as a major component of the innate immunosurveillance of several hematological and solid malignancies. The activity of NK-cells is mainly mediated through their wide variety of receptors with activating and inhibitory functions. Among the versatile receptors present on NK cells, the activating receptor NCR3/NKp30 is a major receptor involved in both direct killing of target cells and mutual NK and dendritic cell activation.

Gastrointestinal stromal tumors (GIST) and Neuroblastoma (NB) are known to be tumors sensitive to NK immunosurveillance. In a recent study we showed that alternative splicing of NCR3/NKp30 gene can affect NK cell function and GIST patient's outcome.

In order to better characterize the GIST tumor-infiltrating lymphocytes, we analyzed the CD3+, T regulatory (Treg) and NK lymphocytes infiltration within primary localized GIST tumors and we determined their prognostic value. We described that, before treatment, NK cells are mainly localized in fibrous trabeculae while T lymphocytes are in the tumor nests in HLA-I positive tumor cells contact. Moreover infiltrating NK cells displayed a secreting $CD56^{bright}$ phenotype, and accumulate in tumor nests after Imatinib (IM) treatment. Importantly CD3+ and NK lymphocytes independently predicted progression free survival (PFS). These results highlight the importance of the immune infiltrate in re-define the GIST risk stratification and allow enhancing the immune response in the therapeutic decisions.

We next investigated the proportions of NK cells in blood and bone marrow (BM) in a cohort of localized and metastatic NB; a high proportion of $CD56^{bright}$ NK cells was associated with metastatic NB and with poor response to induction treatment within the metastatic NB. Moreover, infiltrated BM presented NKp30 down regulation. The expression of the NKp30 ligand, B7-H6, was found on BM neuroblasts, while the soluble protein, sB7-H6 correlated with resistance to treatment. Furthermore the transcriptional status of NKp30/NCR3 dictated the event-free survival rates of HR-NBs

with minimal residual disease post-induction chemotherapy: in particular patients presenting a high proportion of the immunosuppressive isoform (NKp30c) compared to the pro-inflammatory isoform (NKp30b), presented a worse outcome. We further demonstrated the significant role of monocytes to amplify the NKp30 activation response.

These researches in GIST and NB, two different but at the meantime NK-sensitive diseases support the effort to define new immunological therapeutic approaches and to determine their optimal use.

RESUMÈ

Depuis la formulation de la théorie de l'immuno-surveillance en 1957 par Burnet et Thomas, le monde scientifique s'est efforcé d'identifier les cellules immunitaires impliquées dans ce processus. Les lymphocytes Natural Killer (NK) constituent une composant majeure de l'immuno-surveillance innée dans plusieurs cancers hématologiques et solides. L'activité des lymphocytes NK passe principalement par une grande variété de récepteurs avec un rôle activateur ou inhibiteur. Parmi les récepteurs activateurs présents à la surface des lymphocytes NK, le récepteur NCR3/NKp30 a un rôle majeur dans la toxicité directe contre la cellule cible et dans l'activation des cellules dendritiques.

Les tumeurs stromales gastrointestinales (GIST) et le Neuroblastome (NB) sont deux tumeurs sensibles à l'immuno-surveillance par les lymphocytes NK. Dans une étude récente notre équipe a démontré que l'épissage alternatif du gène NCR3/NKp30 peut être déterminant dans la fonction NK et dans la survie des patients atteints de GIST.

Afin de caractériser les lymphocytes infiltrant le GIST, nous avons effectué une recherche visant à analyser l'infiltrat des lymphocytes CD3+, des lymphocytes T régulateurs (Treg) et des lymphocytes NK dans des tumeurs GIST localisés, et corréler ces résultats à la survie des patients. Nous avons mis en évidence que, avant traitement, les lymphocytes NK sont surtout localisés au niveau des fibres trabéculaires qui entourent la tumeur, alors que les lymphocytes T sont localisé à l'intérieur de la tumeur en contact avec les cellules tumorales qui expriment HLA-I.

Nous avons aussi observé que les cellules NK ont un phénotype plutôt $CD56^{bright}$ et migrent à l'intérieur de la tumeur après traitement par Imatinib. L'analyse de survie a mis en évidence que les lymphocytes NK et T peuvent prédire la survie sans progression (PFS). Ces résultats mettent en évidence l'importance de l'infiltrat immunitaire dans la prédiction du risque de rechute dans le GIST et surlignent l'importance de viser une réponse immunitaire dans les protocoles thérapeutiques.

Nous avons ensuite déterminé la proportion de lymphocytes NK dans le sang périphérique et dans la moelle dans une cohorte de Neuroblastome (NB) localisé et

métastatique : une infiltration plus important par les NK CD56^{bright} a été observé chez les patients présentant une maladie métastatique et chez les patients avec une réponse mineure au traitement d'induction. De plus, les NK présents dans les échantillons de moelle osseuse infiltrés par les neuroblastes, présentaient une expression plus basse du récepteur NKp30. L'expression du ligand de NKp30, B7-H6, a été mise en évidence sur les neuroblastes infiltrant la moelle osseuse, et sa forme soluble, sB7-H6, a été retrouvée être positivement corrélée à l'extension de maladie et inversement à la réponse au traitement d'induction.

L'analyse de l'épissage alternatif du gène NCR3/NKp30 a permis de mettre en évidence l'impact des isoformes NKp30 sur la survie sans progression chez les patients atteints de NB de haut risque en maladie minimale résiduelle après chimiothérapie d'induction. En particulier, les patients présentant un taux élevé de l'isoforme pro-inflammatoire (NKp30b) par rapport à l'isoforme immunosuppressive (NKp30c), présentent une meilleure survie sans évènement. Nous avons aussi démontré le rôle des monocytes dans l'amplification de la réponse NKp30 dépendant.

Les résultats de notre recherche dans le GIST et dans le NB, deux maladies différentes mais toutes les deux sensibles aux lymphocytes NK, surlignent l'importance d'intégrer de nouvelles options thérapeutiques aptes à cibler le système immunitaire.

LIST OF ABBREVIATIONS

ADCC antibody-dependent cellular cytotoxicity

BAT3: HLA–B-associated transcript 3

BM : bone marrow

CD cluster of differentiation

cDNA : complementar DNA

c-kit : Stem Cell Factor Receptor (or CD117)

DC dendritic cell

dNTP : desoxyriboNucleotide Tri-Phosphate

DNA : deoxyribose nucleic acid

DNAM-1: DNAX Accessory Molecule-1

EBV : Epstein-Barr-virus

FACS : fluorescence-activated cell sorting

FasL: Fas ligand

FcR: Fc immunoglobulin receptor

Fig : figure

GIST : gastrointestinal stromal tumor

GM-CSF: Granulocyte and Macrophage - Colony Stimulating Factor

G-CSF: granulocyte colony stimulating factor

H : hour

HIV : human immunodefiency virus

HLA : human leukocyte antigen

HR-NB : High risk neuroblastoma

IFNg: interferon- g

Ig : immunoglobulin

IL : interleukin

IM : imatinib

ITAM : immunoreceptor tyrosine-based activation motif

ITIM : immunoreceptor tyrosine-based inhibition motif

lncRNA: long non-coding RNA

KIR : killer cell Ig-like receptor

mAb: Anticorps monoclonal (monoclonal Antibody)

MFI: Mean Fluorescence Intensity

MHC I: major histocompatibility complex class I

MICA (ou B): MHC-class I-related chain A (ou B)

mRNA : messanger RNA

miRNA : microARN

NB : neuroblastoma

NCR : natural cytotoxicity receptor

NK : cell natural killer cell

NKp30L : NKp30 ligand

PBMC: Peripheral Blood Mononuclear Cells

PCR: polymerase chain réaction

PI3K: phosphatidylinositol-3 kinase

PTK: protein tyrosine kinase

qPCR: quantitative PCR

RNA Ribonucleic acid

rs: reference SNP

RT reverse transcription

sB7-H6 : soluble protein B7-H6

SCH : stem cell harvest

SHP SH2 domain containing phosphatase

SNP: Single-Nucleotide Polymorphism

TCR : T Cell Receptor

TGFb : transforming growth factor beta

TLR : Toll-like Receptor

TILS : Tumor Infiltrated Lymphocytes

TNF-α tumor necrosis factor α

TRAIL : TNF-related apoptosis-inducing ligand

ULBP UL16 binding protein

1. INTRODUCTION

1.1 THE TUMOR MICROENVIRONMENT: THE ROLE OF THE IMMUNE SYSTEM

a. The complexity of cancer development

Tumor development and progression depend on several factors according to the type of cancer. However current models try to incorporate heterogeneous biological factors in order to establish basic processes involved in cancer development.

Cancer is due to a multi-step process with successions of genetic transformations of a normal cell with consequent cell growth out of control.

The tumor, formed of these abnormal cells, may remain within the tissue in which it originated (*in situ cancer*), or it may begin to invade nearby tissues (*invasive cancer*) and spread into the blood or lymph to colonize new organs *(metastases)*. [1]. This model is required to have more complex rules: in fact not only genomic aberrations but also an alteration in homeostasis, proliferation and microenvironment interaction are needed.

Hallmarks, as enunciated by Hanahan and colleagues [2], acquired in the neoplastic state are:

- Cancer cells stimulate their own growth by different mechanisms like producing their own growth factors, increasing tumor-associated stroma, expressing constitutively activated growth cell receptors.
- Cancer evades inhibitory signals by genomic mutations with consequent constitutive activation of signaling circuits usually triggered by activated growth factor receptors.
- Cancer invades local tissue and spreads to distant sites: different classes of metastasis genes mediate this multi-steps process.
- Cancer achieves immortality by loss of control on the replication system
- Cancer resists cell death by evading the programmed cell death mechanisms (apoptosis)
- Cancer stimulates the growth of blood vessels to supply nutrients to tumors (auto sustained new angiogenesis)

In the recent vision of tumor development, new emerging recognized hallmarks shared by almost all tumors are[3]:

- Deregulation of cellular energetics: capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation.
- Evasion of immunological control: evading immune surveillance.
- Genome instability and mutation: cancer cells present severe chromosomal abnormalities.
- Inflammation with the evidence that chronic inflammation is linked to the development of many types of cancers.

Cancer, as a multistage process, occurs in the context of different organs with different features, and then researches have to take into account the organ microenvironment and the host (=patient) biological background.

b. The tumor-host interactions: the immune microenvironment

The tumor microenvironment shows a structured organization composed of extracellular matrix, secreted soluble factors (growth factors, proteases) and non-neoplastic host cells, including abnormal fibroblasts, vascular endothelial cells and immune cells. The composition of the tumor microenvironment is highly variable; several differences are seen between patients and often in different areas of the same tumor. Moreover, tumor microenvironment changes its features as the disease progresses [4].

The tumor microenvironment presents distinctive survival conditions, compared to those found in normal tissue, like hypoxia, acidic conditions and low glucose levels. Thus, only the cells with genetic mutations that permit them to survive in severe conditions will continue to grow and contribute to the tumor spreading.

These conditions of growth impact also the normal cells surrounding the tumor that, consequently, exhibit altered characteristics compared to corresponding cells in normal tissue.

Experiments have illustrated the importance of the stroma in tumor development [5]. In direct contact with stroma, immune cells are present to varying degrees. Tumor–host interactions are mediated indirectly through extracellular matrix

molecules and soluble bioactive molecules released from host or neoplastic cells or both, and directly mediated through cell-surface molecules.

The observation of infiltrating inflammatory cells in tumors is an old observation since Virchow in 1863 postulated that cancer is due to a chronic inflammation [6]. This inflammation mainly consists of innate immune system cells (neutrophils, macrophages, eosinophils, dendritic cells, natural killer lymphocytes) but also of adaptative immune response cells (lymphocytes B and T) all of which are capable of producing an assorted array of cytokines and cytotoxic mediators (Fig 1).

Tumor-infiltrating immune cells play dual roles with potential to either eliminate or promote malignancy [7]. Despite exerting a key role in host protection, tumor surveillance by the immune system may eventually fail. As postulated in the theory of cancer immunoediting, tumor cells are initially eliminated by the immune system before becoming clinically detectable. This is then followed by an equilibrium phase, where selection processes for less immunogenic tumor variants take place until the tumors finally "escape" the immune surveillance [8]. On the other hand, the persistent inflammation associated with chronic infections may also encourage new tumor formation.

In the latest years many clinical studies have underlined the link between immunological tumor infiltrations and progression or response to treatment in different types of cancer like breast, ovarian, colorectal, gastric, skin and hepatocellular cancers and many others [9, 10].

In various solid tumors the presence of tumor-infiltrating immune cells correlates with better overall survival but the role of some immune cells like T regulatory lymphocytes and/or macrophages can be controversial [11].

To summarize, the actors of the microenvironment immune infiltrate are:

- **Neutrophils (PMN):** make up a significant portion of the inflammatory cell infiltrate found in a wide variety of human cancers [12, 13]. PNN are a component of the innate immune systems aiding in tumor immune evasion by matrix degradation, immunosculpting, tumor cell proliferation, increased

metastasis, and enhanced angiogenesis. Recent studies demonstrate that PNN can also protect from metastasis progression by matrix metalloproteinase-8 production, which plays a protective role in cancer through its ability to regulate the inflammatory response [14].

- **Macrophage:** Macrophages are a primary source of secreted pro-inflammatory cytokines. These cells can be generally categorized as type 1 (M1) or type 2 (M2). M1 macrophages secrete cytokines such as interleukin 12 (IL-12) and can assist in the generation of T-helper 1 (Th1) adaptive immunity and communicate a direct cytotoxic effect to tumor cells. M2 macrophages secrete immunosuppressive cytokines and promote tumor cell growth and neoangiogenesis [15]. Tumor-associated macrophages (TAMs) are, in general, of the M2 phenotype, and infiltration by these cells has been shown to be an independent predictor of poor prognosis in multivariate analysis in many malignancies [16].

- **Monocyte/Myeloid-derived suppressor cells** (MDSCs): MDSCs are a component of the innate immune cells. MDSCs promote cancer growth by blocking the adaptive immune response via the direct secretion of substances that affect T-cell function as well as the induction of adaptive T regulatory (Treg) cells [17]

- **T Lymphocytes:** several studies have demonstrated the role of T lymphocytes in controlling tumor growth. CTL (cytotoxic T lymphocytes), able to produce IFNg and to eliminate cancer cells, are mainly CD8+ T cells while CD4+ lymphocytes (T-helper) participate in the tumor micro-environment, both in enhancing tumor growth (immunosuppression), and in enhancing CTL activity [18].

  Treg (regulatory T cells) are CD4+CD25+ lymphocytes expressing the transcriptor factor FoxP3 [19] with a suppressive capacity on regular T lymphocytes function. Many studies have correlated increased Treg presence with disease progression [20].

  In the family of CD4+ lymphocytes, Th17 lymphocytes display a great degree of context-dependent plasticity. Th17's role in cancer immunity is ambiguous [21]

but experiments involving adoptive cell transfer therapies have demonstrated their an antitumor activity [22].

Vα24-invariant NKT cells are an evolutionarily conserved sub-lineage of T cells characterized by reactivity to self- and microbial-derived glycolipids presented by an HLA class-I–like molecule, CD1d. Several studies have revealed strong positive associations between the numbers of tumor infiltrating or circulating NKTs with improved disease outcome in patients even with diverse types of CD1d-negative solid tumors [23].

- **B Lymphocytes:** are essential for establishing chronic inflammatory states that are associated with pre-malignant lesions. Their role in tumor control is controversial because B cells can enhance T cell responses by producing Abs, stimulatory cytokines and serving as APC [24] while, on the other hand, B-cells can promote tumor growth [25] by a regulatory immunosuppressive pattern.

- **Dendritic cells (DC):** Although DCs are notoriously known to be the most potent T cell activating cell type, their function as antigen-presenting cells (APC) is markedly compromised in the cancer microenvironment. DC might be also polarized into immunosuppressive/tolerogenic regulatory DC, which limits the activity of effector T cells and supports tumor growth and progression [26] .

- **Natural Killer cells** are lymphocytes at the border between innate and adaptative immunity; they play an important role in the innate immune response against cancer. Several studies highlight their role in the elimination of tumor metastases and small tumor[27]. Natural killer lymphocytes characteristics and functions will be detailed in the next chapter.

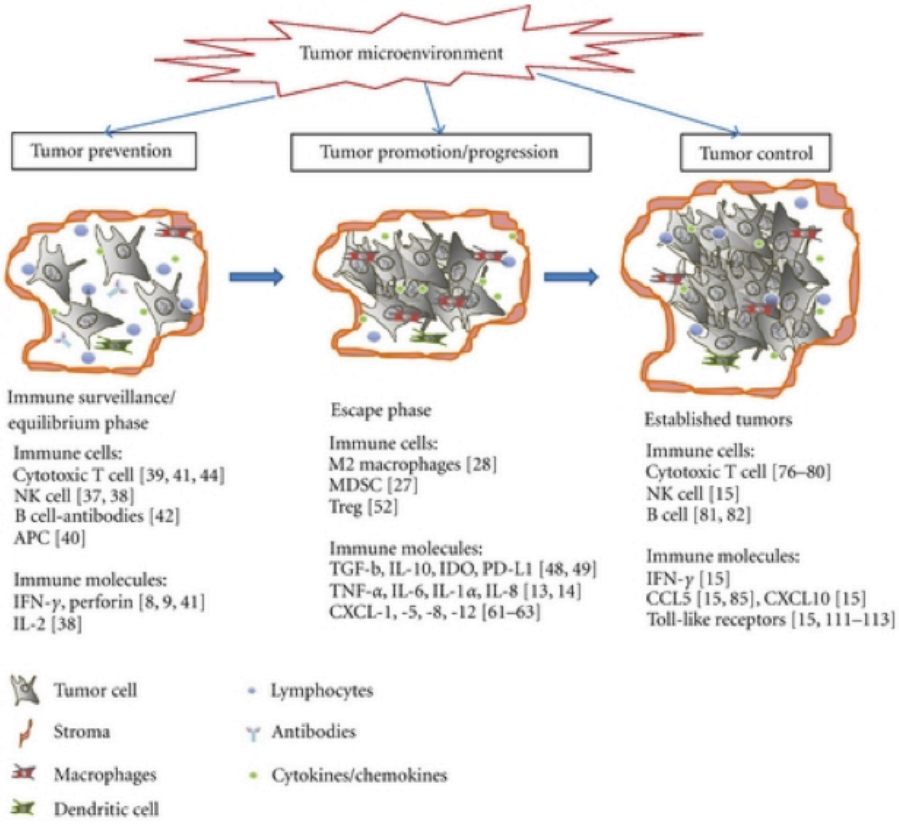


Fig 1. The immune roles in cancer development (from Chew V et al Journal of Oncology 2012)

1.2 NATURAL KILLER LYMPHOCYTES (NK)

a. Characteristics and physical parameters of activation

NK are a component of the innate immune system defined "killers" due to their capacity to kill tumoral, infected or stressed cells without the requirement of priming by antigen presenting cell (APC) in contrast to cytolytic T cells [28, 29].

NK cells are large granular lymphocytes, with cytoplasm enriched of lytic granules. They are highly conserved in the phylogenesis because cytolitic cells have been part of the immune defense system approximately 500 million years ago[30]. NK, which are relatively short lived cells renewing each 2 weeks [31] are present in the blood, where they represent 5-15% of total lymphocyte population; in secondary lymphoid organs like lymph nodes, spleen and tonsils and in some organs like lung, liver and placenta.

Human NK cells are defined within the lymphocyte gate on the flow cytometric analyzer as CD3 negative and CD56 positive (which is a neuronal cell adhesion protein) cells.

Different functions are known for CD3-CD56^{bright} cells and CD3-CD56^{dim} cells: while the bright ones are able to produce large amount of cytokines but not to kill tumor target, NK CD56^{dim} can kill some tumor target but are less able to produce cytokines and chemokines after activation. In a healthy adult, circulating NK cells are mostly with a CD56^{dim} phenotype. The majority of human NK cells are CD56 ^{dim} and express high levels of the FcγRIIIA, low affinity receptor for the Fc portion of immunoglobulin G (CD16).

b. NK cells development

NK cells are derived from a CD34+ hematopoietic stem cell and they do not need an intra-thymus development like T lymphocytes. The exact development of NK cells is still not really known but interaction with some soluble factors and receptors in bone marrow (like c-kit ligand, Flt3 and IL-15) are needed. The development occurs not only in the bone marrow but also in secondary lymphoid organs where CD56^{bright} NK (immature) could be found [32]. During the maturation process, NK cells

differentiate into highly proliferative CD56^{bright} cells. Terminal differentiation involves a down-regulation of CD56, changes in the receptor profile and acquisition of cytotoxic function [33]. In the meantime, NK cells are "educated" by recognition of MHC class I molecules in developing inhibitory receptors, which are found in CD56^{dim} NK. Cytokines such as IL-15, IL-2, and IL-21, are involved in NK cell development and acquisition of effector functions, in particular, IL-15 is required for the maturation and survival of NK cells [31]. The high affinity receptor for IL-2 is mostly expressed on the CD56^{bright} subset.

c. NK functions and subsets

NK cells are cytolytic effector lymphocytes, which, unlike cytotoxic T cells, can directly induce the death of tumor cells and virus-infected cells in the absence of specific immunization via perforin/granzyme production or death receptor (Fas ligand, TNFa, TRAIL pathway), and moreover the NK CD56^{bright} cells are a major producer of IFNg, which is the key to activate naïve T lymphocytes in the lymph nodes [34]. But NK cells also produce many other cytokines with both pro inflammatory and immunosuppressive functions, such as tumor necrosis factor–α (TNF-α) and interleukin 10 (IL–10), respectively, and growth factors such as GM-CSF (granulocyte macrophage colony-stimulating factor), and IL-3, in this way they can positively [35, 36] or negatively [37] influence host T and B immunity.

The CD56^{bright} NK subset generally requires 2 signals to produce IFN-g: the IL-12 released by monocytes, macrophages and DCs and the cytokines IL-1, IL-2, IL-15 or IL-18, or the engagement of an NK activating receptor [38]. The CD56^{dim} NK lymphocytes are the one able to directly kill a target cells through a mechanism called antibody-dependent cellular cytotoxicity (ADCC). This subset mainly expresses CD16 (Fcg receptor IIIA), which can bind to the constant (Fc) region of immunoglobulin when they are immobilized on the cell surface of a target cell. This receptor-ligand binding is followed by a CD16-mediated activation signal that results in NK-cell degranulation and perforin dependent target cell lysis.

CD56^{bright} and CD56^{dim} completely differ for their chemokine receptors and adhesion molecules repertoire (Fig. 2): while the bright subset express CCR7, CXCR3, CD2,

CD11c, CD44, CD49e, CD54 and CD62L, the $CD56^{dim}$ NK express CXCR1, CX3CR1 and CD11a. This different repertoire induces different migratory properties: the $CD56^{bright}$ preferentially migrates to secondary lymphoid organs whereas the $CD56^{dim}$ cells migrate to the acute inflammatory sites. Moreover, $CD56^{bright}$ NK cells express high levels of the inhibitory CD94/NKG2A complex recognizing HLA-E, but lack KIRs receptors which are, in contrast expressed by $CD56^{dim}$ NK cells.

As mentioned before, NK cells may also play immuno-regulatory functions mainly by the IL-10, IL-21 and HLA-G production. Experimental in vitro produced "regulatory" NK cells [39] present a CD56+, CD16+, NKp30+, NKp44+, NKp46+, CD94+, CD69+, CCR7+ and are able to down-regulate the immune response. Interestingly, the $CD56^{bright}$ NK cells produce high amounts of the immunosuppressive cytokine IL-10. In mouse model, tumor derived IL-18 is able to expand a subset of NK cells that express the c-Kit receptor [40]. These NK cells regulate innate NK cell functions. Up to now, this NK subset has not been yet identified in human.

In the sites of peripheral inflammation, $CD56^{bright}$ also could be found (40%) with an activated phenotype (CD69+). Upon stimulation by IL-12, IL-15 and IL-18, $CD56^{bright}$ NK cells produce IFNγ and increase the production of TNFα from monocytes.

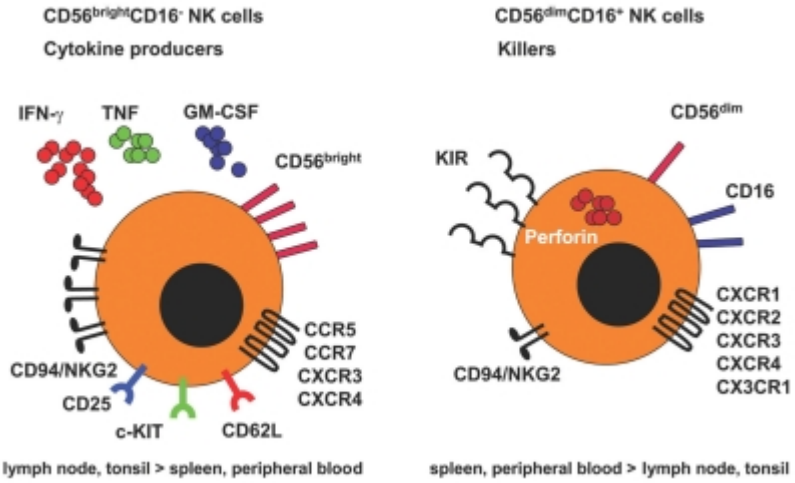


Figure 2. Characteristics of NK subsets ($CD56^{bright}$ and $CD56^{dim}$), (From Lunemann et al 2009)

d. NK cell Receptors

The main characteristic of NK cell regulation is the protection of self-cells (normal) and to destroy the non-self (transformed) cells. The machinery to effectuate this regulation includes: a) recognition of "missing self" by inhibitory receptors when the inhibitory ligands (self proteins-MHC I) are down-regulated like in infected or transformed cells [41] b) recognition of ligands expressed on "stressed" or transformed cells by NK activating receptors[42]. These NK receptors are shown in Fig 3.

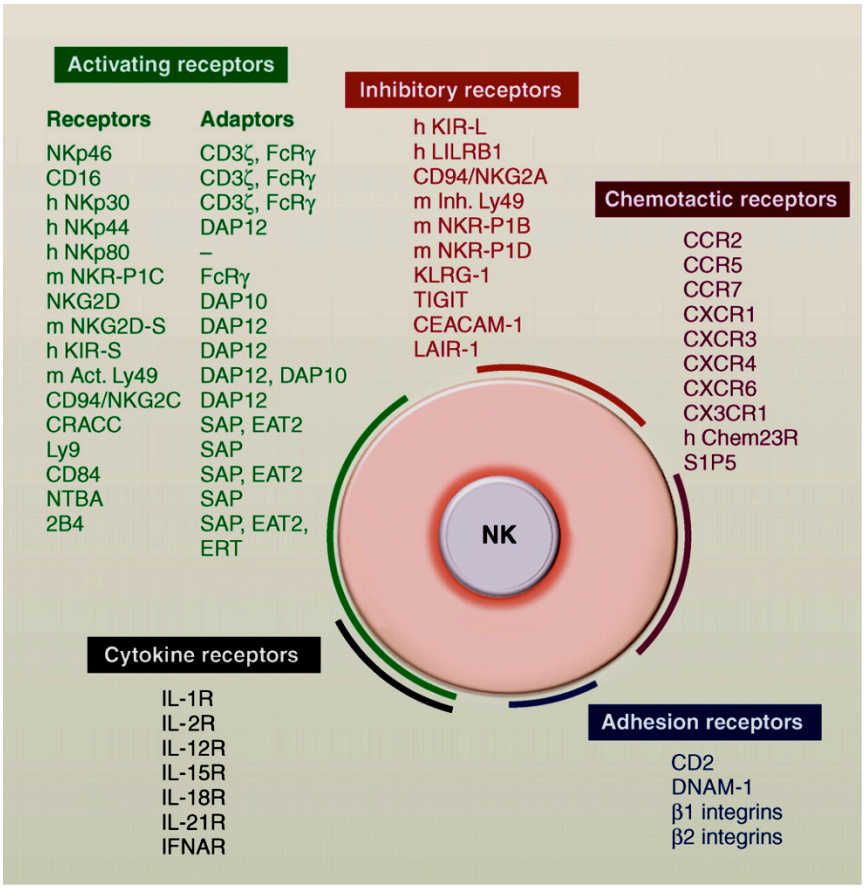


Fig 3. Natural Killer Receptors (from Vivier E et al Science 2011)

Inhibitory Receptors

Normal cells are characterized by the presence of self-antigens like MHC-class I molecules and the recognition of MHC class I molecules by NK inhibitory receptors dominates over the activation signals and blocks the effector functions of NK cells.

The receptors responsible for this inhibitory function include the MHC class I specific killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A.

KIR receptors recognize the polymorphic MHC class I molecules. The inhibitory signal results from the presence of the immunoreceptor tyrosine-based inhibition motifs (ITIM) in the cytoplasmic domain of the inhibitory receptors. The phosphorylation of ITIMs upon MHC I-ligand engagement of inhibitory receptors results in the recruitment and specifically binding of Src-Homology-2 (SH-2) domain containing protein phosphatases. Activated phosphatases such as SHP-1 and -2 are able to dephosphorylate multiple targets in the activating pathway, thereby mediating its negative signaling. As a result, the activating receptor signaling is directly inhibited by the de-phosphorylation of ITAM-recruited protein-tyrosine kinases[43].

The different inhibitory KIR receptors with the correspondent ligands are depicted in Fig 4.

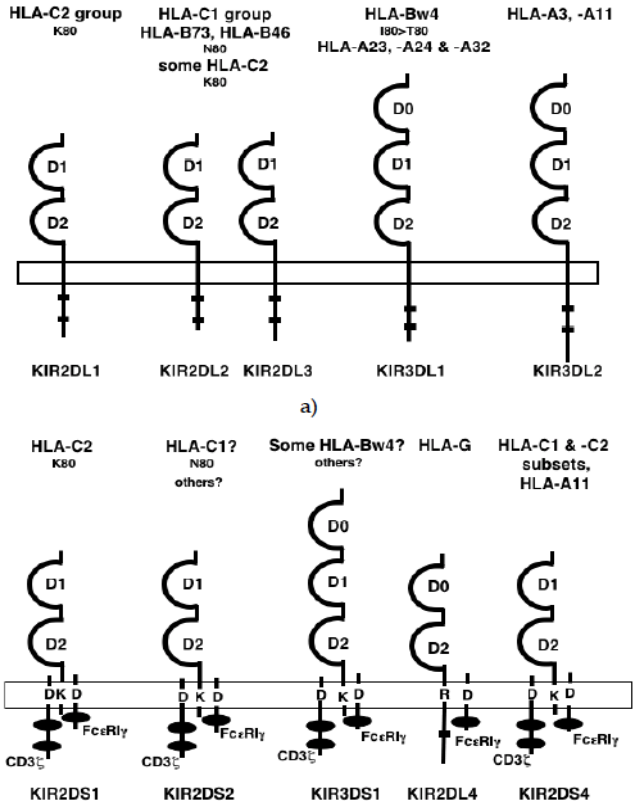


Fig 4. KIR receptors and HLA-I ligands (from Biassoni R et al, Intechopen)

Other inhibitory receptors are:

- CD94/NKG2A, which recognizes the non-classical class I molecule HLA-E, with a consequent inhibitory ITIM signal
- CD161 (KLRB1)
- ILT2 (Immunoglobulin like transcript 2), which recognizes classical HLA-I and HLA-G but also CMV MHC-I homologue.
- LAIR-1 (CD 305) which recognizes Ep-CAM
- P75 and IRp60

Activating NK Receptors

NK activating receptors are necessary for the initial activation of NK cell effector functions. These are non-covalent associated with transmembrane-anchored signaling adaptor proteins like CD3ζ, FcεRIγ, DAP10 or DAP12. The engagement of activating receptors activates "first line" protein tyrosine kinases (PTKs) of the Src-family, which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic tail of the adaptor proteins. Recruitment and activation of "second line" PTKs of the Syk-family like Syk and ZAP70 then results in the initiation of the downstream signaling cascade.

NK activating receptors are non-MHC specific receptors and include: low affinity Fc receptor FcγRIII (CD16), antibody activating receptor; NKG2D, a triggering receptor; the Natural Cytotoxicity Receptors (NCRs: NKp46/NCR1, NKp44/NCR2, and NKp30/NCR3) and the activating homologs of inhibitory MHC class I receptors (members of the Ly49, KIR and NKG2 families) [44].

CD16: NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) by expressing a low affinity Fc receptor FcγRIII (CD16). This receptor possesses ITAM motifs and, upon ligation, activates src-family tyrosine kinases (eg. Lck) and phosphorylates tyrosine residues contained within the ITAM motifs [45, 46]. This signal activates NK cells which results in secretion of cytokines, and antibody-

dependent cellular cytotoxicity (ADCC) leading to apoptosis as a consequence of Fas-ligand induced cell death [47-50].

NKG2D: NKG2D is a type II disulphide-linked dimer with a lectin like extracellular domain which requires the association with the adaptor subunits DAP10 (DAP10 or DAP12 in mice) that mediate signaling, since the intracellular domain of NKG2D has no signaling motifs. NKG2D is expressed by CD8+ αβ-T cells (but also CD4+ in some tumors, eg melanoma), by almost all human γδ-T cells and human and murine NK cells. [51]

In human NK cells the triggering of NKG2D induces cytotoxicity but no cytokine release. Importantly, since NKG2D has a downstream signaling pathway that is distinct from the activating KIR and C-type lectin receptors, the triggering via NKG2D is less susceptible to blocking by KIR- or NKG2A generated inhibitory signals. As a consequence, the signaling through human NKG2D was postulated to override inhibition signals generated by MHC class I engagement, and thus NKG2D functions as a primary cytotoxicity receptor rather than a co-receptor [52, 53].

The ligands for human NKG2D are the MHC class I chain related proteins A and B (MICA/B) and the UL16 binding proteins ULBP-1, -2, -3 and -4. In normal tissue low levels of MICA/B are found mainly on epithelial cells and fibroblasts. MIC molecules are highly polymorphic since at least 50 different MICA and more than 15 MICB alleles are currently known [54]. MICA/B are glycoproteins that contain MHC-like α1-, α2- and α3-domains but, in contrast to MHC class I molecules, do not require β2-microglobulin or peptides for stable surface expression. The family of the UL16-binding proteins (ULBPs) is NKG2D ligands that are glycophosphatidyl inositol (GPI)-linked surface molecules, which initially were identified by their ability to bind to the human CMV-derived membrane glycoprotein UL16 [53].

Induction or up-regulation of NKG2D ligands may occur with pathogen related cellular stress, viral infection or tumor cell transformation. High MICA and MICB expression was found on epithelial tumors and on CMV infected epithelial tissues

or fibroblasts. Indeed, the induced expression of NKG2D ligands were shown to markedly enhance the sensitivity of tumors to NK cells in vitro and in vivo. In Neuroblastoma and other cancers, a soluble form of MICA (sMICA) was identified in patient sera derived from metalloprotease-mediated proteolytic shedding from the tumor cell surface: sMICA impairs NKG2D-mediated immune surveillance of tumors by triggering internalization of surface NKG2D [51].

Natural Cytotoxicity Receptors (NCRs): these are three Ig-like molecules termed NKp46 (CD335), NKp44 (CD336) and NKp30 (CD337). NKp46 and NKp30 are present both in resting and activated NK cells, whereas NKp44 is acquired only on activation. NCRs are characterized by two Ig-C2 (NKp46) [55] or one Ig-V (NKp30 and NKp44) [56, 57] domains in the extracellular portion. Their short cytoplasmic tail lacks the typical tyrosine-based activating motifs but instead the trans-membrane regions contain positively charged amino acids that allow association of ITAM- bearing polypeptides, CD3ζ and FcεRIγ for NKp46 and NKp30, while NKp44 associates with DAP12[58, 59]. Interestingly, NCR, instead of representing individual receptors, appear to form a molecular complex because both their expression and functions are coordinated [60]. NCR 's ligands are not completely known: virale hemoagglutinin (HA) and vimentin expressed on Mycobacterium tuberculosis-infected human monocytes are known to be NKp46 ligands, NKp46 could recognizes different type of tumoral cells and neutrophils; NKp44 cans also recognize HA and Mycobacterium but also Pseudomonas Aeruginosa; the known ligands for NKp30 are B7-H6 and BAT-3 molecules which are present mostly in stressed or tumoral cells [61](see next paragraph) (Fig 5).

Co-receptors

The function of NCR is supported and enhanced by the simultaneous engagement of different co-receptors (Fig.4). These include non-restricted surface molecules such as 2B4 (CD144), NTBA, NKp80 and CD59.

2B4 and NTBA are members of the CD2 sub-family of the immunoglobulin superfamily. Whereas in normal NK cell engagement of both coreceptors results in triggering cytotoxicity, they transduce inhibitory signals in NK cells derived

from X-linked lymphoproliferative disease (EBV infected cells). NKp80 participates in NK-myeloid cells cross talk; its ligand is AICL (activation induced C-type lectin). Another frontier-line co-receptor is DNAM-1 (CD226), which is an activating molecule that recognizes poliovirus receptor and Nectin -2 (highly expressed by different tumors)[44].

Receptors	Ligands
Activating/inhibitory Receptors	
FcγRIII (CD16)	Fc of antibodies
CD2	CD58 (LFA-3)
LFA-1	ICAM-1
2B4	CD48
CD69	Unknown
DNAM-1 (CD226)	CD112, CD155
NKp80	AICL
Tactile (CD96)	CD155, CD111
TIGIT	CD112,CD113,CD155
CRTAM	TSLC1
C-type Lectin receptors –Activating/Inhibitory	
CD94/NKG2A/B	HLA-E
NKG2D	MICA, MICB, ULBP-1, ULBP -2, ULBP -3, ULBP -4, ULBP -5, ULBP -6
CD94/NKG2C	HLA-E
CD94/NKG2E/H	HLA-E, Qa-1b
Natural cytotoxicity receptors (NCR)	
NKp46 (NCR1)	Viral Hemagglutinin
NKp44 (NCR2)	Viral Hemagglutinin
NKp30 (NCR3)	B7h6, HCMV-pp65
Killer IG-like (KIR) – Activating/Inhibitory	
KIR2DLs, KIR3DLs, KIR2DS	HLA-C, HLA-B, HLA-A, HLA-G
Cytokines, growth factors and chemokines	**Cytokines, growth factors and chemokine ligands**
Toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR)	**Bacterial DNA, LPS, peptidoglycan, teichoic acids, flagellin, pilin, viral dsRNA and fungi zymosan**

Fig 5. NK receptors and their ligands (from Jewett et al, Journal of Cancer 2013)

e. The NKp30 (CD337) receptor

At the end of 1990s Pende and colleagues identified NKp30 as a novel 30-kDa triggering receptor expressed by all resting and activated human NK cells [57].

It is a type I trans-membrane protein characterized by a single V-type immunoglobulin (Ig) extracellular domain. The intracellular tail of NKp30 has no

signaling motif but its trans-membrane domain associates via a charged amino acid with immune-receptor based activation motif (ITAM)-bearing adapters (CD3ζ and FcRγ). Activation of NKp30 receptor by its ligand induces activation of the NFkB canonical pathway [62]. A recent study [63] identified the flexible stalk region of NKp30 as an important module for ligand recognition and related signaling of the corresponding full-length receptor proteins. In the same study region of glycosilation of the ectodomain of NKp30 is responsible of differential binding affinities and signaling capacities.

NKp30 encoding gene (NCR3) is located in the class III region of the human MHC on chromosome 6 (locus 6p21.3), where several other genes with immune function are found (TNFa eg). Phylogenic analysis of NKp30 in different mammalian species shows that gene sequences are very conserved except in exon 4 that encodes the intracellular domain[64].

NCR3 encodes six alternative spliced transcripts, 4 of these encode for a protein. NKp30a, NKp30b and NKp30c produce a V-type Ig extracellular domain and NKp30d, Nkp30e and NKp30f produce a C-type one. The NKp30 a, b and c are the most represented in tissues. Due to the NCR3-exon 4 alternative splicing, these extracellular domains are coupled with three different intracellular domains (Fig 6).

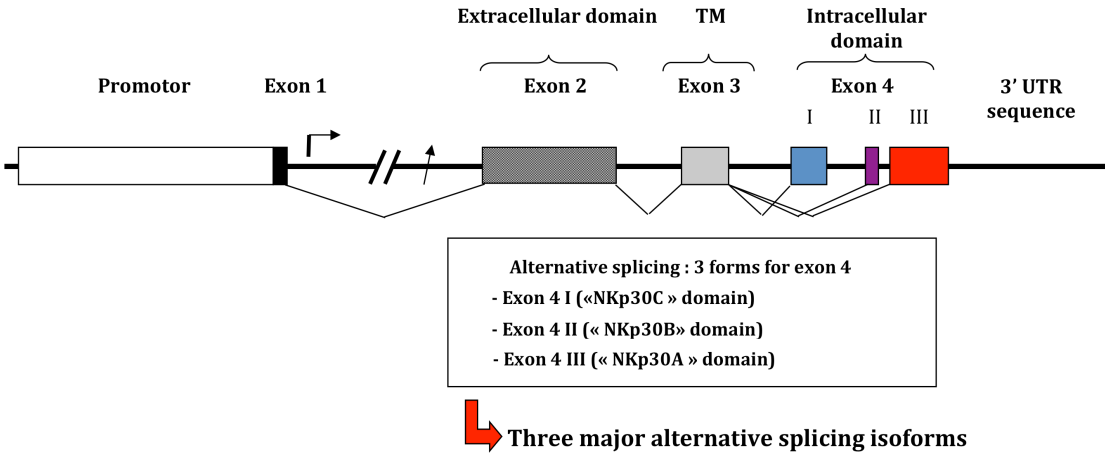


Figure 6 Structure of the NCR3 / NKp30 gene

We demonstrated [65] (see supplemental article 1) that the three different isoforms Nkp30a, NKp30b and NKp30c, issues from the exon 4 alternative splicing in the

intracellular domain, transmit distinct signals and thus mediate different cell functions: NKp30a and NKp30b are immunostimulatory isoforms that trigger TH1 cytokine production while NKp30c promotes IL-10 secretion, relaying an immunosuppressive signal (Fig 7).

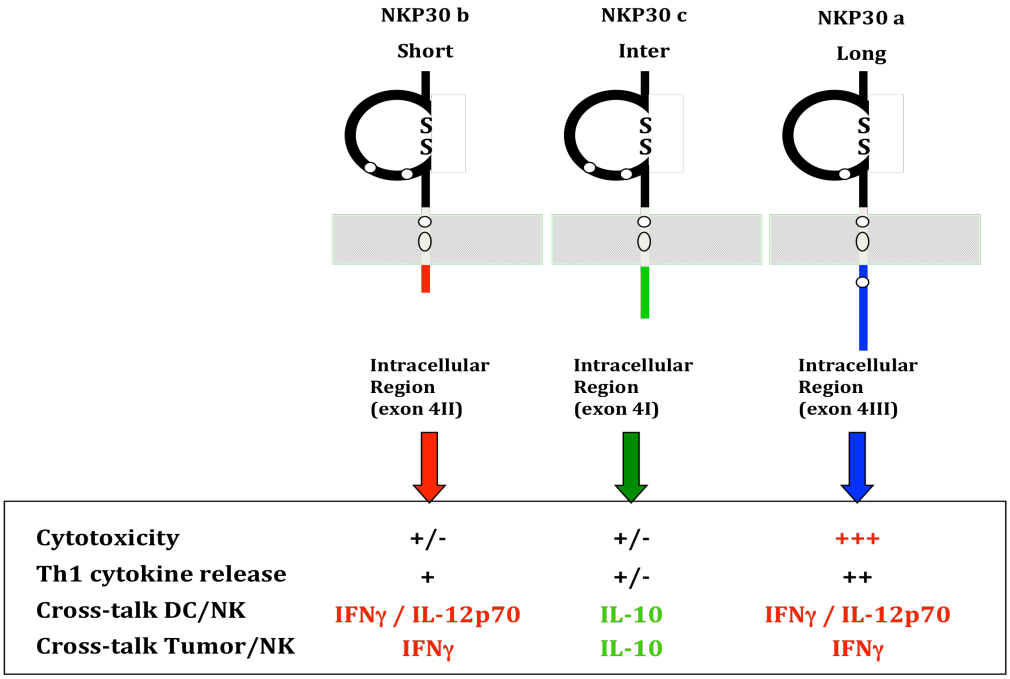


Figure 7. NKp30 isoforms significant differ their functions in vivo

Ligation of NKp30 triggers the canonical pathway of nuclear factor-κB (NF-κB) activation. NKP30C fails to activate NF-kB pathway while induce a rapid phosphorylation of p38 MAP kinase.

NKp30 is the only receptor involved both in tumor cell lyses and lyses of normal cells [57, 66, 67]. It is mostly restricted to NK cells but it could be expressed also on umbilical cord blood T cells after IL-15 stimulation and on endometrial epithelium after progesterone exposure.[68] IL-2 can enhance NKp30 surface protein expression while TGFβ can reduce the expression.

A striking feature of NKp30 is its involvement in the crosstalk between NK cells and DCs. NK cells are able to lyse immature DCs through engagement of NKp30 despite MHC I expression, but mature DCs are protected from lyses [66]. Additionally, NK cells that have been stimulated through NKp30 can produce tumor necrosis factor α (TNF-α), which then induces maturation of DCs [67]. DCs in return produce cytokines like IL-12 or IL-18 that can stimulate NK cells [42]. The mutual control and regulation

of DCs and NK cells again is an interesting example of how cells of the immune system communicate and work together in numerous ways; NK cells and DCs through their communication among each other are crucial in bridging the innate and the adaptive immune response.

In contrast to NKp44 and NKp46, NKp30 has not been linked to the binding of viral hemagglutinins. However, NKp30 has been implicated in the recognition of several pathogens:

- Cytomegalovirus: the CMV tegument protein pp65 bind NKp30 but this engagement leads to an inhibition of NK ability to kill. [69]
- Trypanosoma brucei infected erythrocytes [70]
- Filovirus infected DCs : NKp30 is able to recognize virus like particles of the filovirus type and activate NK cells to produce cytokines [71].

Others known ligands for NKp30 are BAT3 and B7-H6.

BAT3 or HLA-B associated transcript-3 is found among the genes of the MHC cluster, specifically in the MHC III region but it does not show any homology to the common MHC proteins. The intracellular BAT3 protein was found to bind and activate NKp30 [72]. This protein could be exposed on surface or secreted or expressed on exosomes from mature DCs [73] after stress or heat shock. In this way BAT3 regulates the NK cell mediated cytotoxicity against immature DCs.

B7-H6 is a novel member of the B7 protein family (co-stimulatoty and co-inhibitory transmembrane proteins that contribute to the immune response regulation) expressed by certain tumor cell lines and a subset of primary tumor cells (melanoma and carcinoma) or hematological tumors, (lymphoma and leukemia) but not by healthy cells [74]. The protein structure is similar to other members of the B7 family and, particularly, B7-H6 is a PD-L1/B7-H3 homologue. Membrane-bound B7-H6 activates NK cells, leading to degranulation and IFNg secretion. B7-H6 has two extra-cellular Ig domains encoded by exons with adjacent phase 1 introns and an intracytoplasmic domain, which contains signaling motifs like ITIMs. This suggests that, upon engagement, B7-H6 could induce a response in the NK cell target through interaction with intracellular signaling proteins. The absence of B7-H6 transcripts in normal tissues and presence in tumor cell define B7-H6 as a novel example of stress induced self-molecule. Little is known about expression modulation by tumor editing or by microenviroment. Matta et al recently demonstrated

that B7-H6 could be induced on peripheral monocyte in patients with gram-positive septic shock [61].

f. Natural Killer cells and cancer

Several studies have demonstrated the role of NK cells in the control of solid and hematological malignancies. Reduced relapse rate is observed in allogenic stem cell transplantation when there is a mismatch of NK inhibitory receptors between the host and the graft [75]. Defects in NK-cell cytotoxicity have been observed in all hematological malignancies [76, 77].

Numerous tumor models have also demonstrated the key role of NK in solid malignancies: the first one is the demonstration of the importance of NK in controlling tumor in beige mice with defective cytotoxicity against tumor cell lines [78]. Other studies conducted on mice models, have demonstrated the role of NK in controlling spreading metastasis [79] [80, 81]. Several reports have indicated that in mice deficient for activating NK cell receptors, such as NKG2D, NKp46, or DNAM-1 tumor growth was enhanced [82, 83] while an over-expression of the their ligands induces mechanisms of tumor immune-escape.

These results suggest that these NK receptors are involved not only in tumor immune surveillance but also in immunoediting and tumor escape. Accelerated tumor progression was also observed in mice lacking molecules involved in NK cell effector responses, including perforin, TRAIL and IFNg.

Many translational research studies confirmed the role of NK cells in controlling tumor development: in lung, gastric or colorectal cancer patients, a high number of NK cells in the tumor tissue correlated with an improved prognosis [84] [76]. Tumor tissues are preferentially infiltrated by CD56 bright CD16– NK cells. CD56 bright and CD56 dim subsets are differentially recruited to the tumor site and/or whether their phenotype is changed by the tumor microenvironment. Elevated numbers of CD56 bright NK cells in the tumor might be a consequence of their increased proliferation and/or survival. NK/tumor cell ratio is often low and NK cell are not in direct contact with tumor cells but mostly in peritumoral stroma [85, 86].

Many NK receptors are involved in anti-tumor response:

1. The KIRs [87] and CD94/NKG2A heterodimers [88] participate in the regulation of NK activation by tumor cells.
2. NKG2D plays an important role in NK tumor immune-surveillance:

- The expression of their ligands (MICA/MICB, ULBP) is present in many tumors (ovarian, melanoma, breast, colorectal cancer and others) [89, 90]
- NKG2D haplotypes confer different NK cytotoxicity and dictate cancer immune surveillance [91]
- Tumors decrease NKG2D ligand expression via shedding of the extracellular domain by metallo-proteases producing soluble ligands that are detected in some cancers [92, 93] and represent an immune escape way.

3. NCRs also are implicated in the clearance of a variety of tumors like carcinomas, melanoma and neuroblastoma. Impact of NKp30 isoforms in gastrointestinal stromal tumors it will be discussed in the next chapter and in the related supplemental article 1.

   The NKp30 ligand B7-H6 described before is absent in normal cells but it's expressed in hematological malignancies and others tumor cell lines like lymphoma, melanoma and carcinoma.

   The known NKp44 ligand proliferative cell nuclear antigen (PCNA) is highly associated with malignancy and induces an NKp44 inhibitory signal.
4. DNA damage pathway activation increases the expression of the poliovirus receptor CD155 and Nectin-2, ligands for activating receptor DNAM-1 [94]. The simultaneous blockade of DNAM-1 and NCRs often leads to the complete abrogation of tumor cell lysis.

The activation stimuli and the tumor microenvironment determine the type of response exerted by tumor-infiltrating NK cells and this could impair NK activity against tumor. Clinical evidences showed that a decreased NK cell response is detected in patients with lung and liver cancer as well as in melanoma, chronic myeloid and acute lymphoid and myeloid leukemia [95-97]

To date, it is still not clear, which NK cell subpopulation is most effective in controlling tumor growth in vivo. NK cells in the tumor site interplay in tumor with different immune cells: DCs, which often show in tumors an immature phenotype; tumor associates macrophages (TAMs) with an immunosuppressive role, IFNg secreted by

activated NK can prevent macrophage polarization toward a M2 phenotype (immunosuppressive one); Treg, which can interact with NK cells with down-regulation of NK activating receptors (NKG2D) and suppression of IL-12-induced IFNg production by NK cells.

Several additional soluble factors are produced within the tumor microenvironment, including non-classical HLA molecules (HLA-E, -F and -G) and soluble NKG2D ligands [98]. HLA-G expression on malignant cells is controlled by epigenetic mechanisms, hypoxia and cytokines such as GM-CSF, IFNg, IL-10 and LIF [99]. Engagement of KIR2DL4 on NK cells by soluble HLA-G results in the induction of proinflammatory and proangiogenic responses [100].

In this study we will focus on the role of NK cells in two immunological-controlled tumors: Gastrointestinal Stromal Tumors (GIST) and Neuroblastoma (NB).

1.3 GASTROINTESTINAL STROMAL TUMOR (GIST)

a. Characteristics of the disease

Gastrointestinal stromal tumors (GIST) were first described in 1983 by Mazur and Clark as a group of tumors that were non-epithelial in origin, lacked smooth muscle cell ultrastructural features, and did not express immunohistological characteristics of Schwann cells [101].

GISTs are sarcomas of the gastrointestinal tract (mesoderm embryonic origin) and represent more than 18% of all sarcomas [102]. Most GISTs arise from the stomach (60%) or small intestine (25%), but may also arise from the colon and rectum (10%), and, less frequently, from the esophagus, omentum, mesentery, and the retroperitoneum. In GIST the most common sites for metastasis are the liver and the abdominal membranes (peritoneum, mesentery, omentum). GIST rarely spreads to lymph nodes, but it may occasionally affects local abdominal lymph nodes. Unusual sites of metastasis include lung, bone tissues and pelvic sites such as the ovaries[103-105]. Extremely rare sites of metastasis include breast and muscle tissue [106]. Less than 5% of these tumors present a multifocal localization at diagnosis and about half of patients are metastatic or unresectable at the time of presentation [107]. The median age of GIST diagnosis is 58 years; GIST is rarely diagnosed in children with only ~3% diagnosed before the age of 21.

GIST patients have often no symptoms at diagnosis and they are found incidentally during medical imaging for other purposes or during surgery for other conditions. Sometimes symptoms include: digestive discomfort, sensation of abdominal fullness or abdominal pain according to the localization of the tumor.

GISTs are thought to arise from the interstitial cells of Cajal (ICC), pacemaker cells that control gut motility, and this supported by a genomic research that shows that GISTs display a distinct gene expression profile from that of other soft tissue sarcomas. Cajal cellls have immunophenotypic and ultrastructural features of both smooth muscle and neuronal differentiation in varying degrees and serve to regulate peristalsis.

Immunohistochemically, 95% of GISTs stain positively for the KIT protein, irrespective of the site of origin, histological characteristics, or biology of the individual tumor, making KIT expression a key diagnostic marker of the disease [108]. In addition GIST cells express CD34+ antigen (60-70% of GISTs). Protein kinase C (PKC)-theta, an isoform of PKC, is highly expressed and useful to identify the rare subset of GISTs that do not express KIT (~5%) [109]. A proper diagnosis of GIST is often only reached through a careful analysis of pathological and clinical findings.

b. GIST staging system

Many efforts for the stratification of the risk of relapse in GIST have been made over time. The earliest risk-stratification schemes or GIST was developed with the National Institutes of Health (NIH) in 2001 and estimates metastatic risks based on tumor size and mitotic count [110]. To integrate the risk linked to the localization of the tumor, Miettinen and colleagues created the Armed Forces Institut of Pathology (AFIP) risk table (Fig 8)[108].

More recently the American Joint Committee on Cancer (AJCC) has developed another classification to integrate the AFIP risk with the TNM (tumor, lymph nodes and metastasis) system. Staging is different for gastric and omentum GISTs versus other GISTs, reflecting a greater risk of recurrence for non-gastric GISTs [111].

With the new integrated factors of risk (tumor rupture, localization) patient at low risk are considered to be those who have 10% or less likelihood of recurring at 5-years. Patients at high risk are those who have 30% or more chance of recurring at 5 years. Anything between 10% and 30% is considered an intermediate risk [112].

Mitotic Index	Size	Gastric	Jejunal/Ileal	Duodenal	Rectal
≤5 per 50 high-powered fields*	≤2cm	0%	0%	0%	0%
	>2cm to ≤5cm	1.9%	4.3%	8.3%	8.5%
	>5cm to ≤10cm	3.6%	24%	34%[c]	57%[c]
	>10cm	12%	52%		
>5 per 50 high-powered fields*	≤2cm	0%[b]	50%[b]	Insufficient data[d]	54%
	>2cm to ≤5cm	16%	73%	50%	52%
	>5cm to ≤10cm	55%	85%	86%[c]	71%[c]
	>10cm	86%	90%		

**A high-power field approximates to 0.2 mm². [a]Metastasis or tumour-related death. [b]Small number of cases. [c]Risk combined due to small number of cases. [d]No cases in this category were included in the study. Note that small intestinal and rectal GISTs show a markedly worse prognosis in many size/mitotic index categories compared with gastric GISTs. Adapted from Miettinen M, Lasota J. Seminars in Diagnostic Pathology 2006;23:70-83.*

Key: No Risk | Very Low Risk | Low Risk | Moderate Risk | High Risk

Fig 8. Risk of progressive disease in GIST (adapted from Miettinen and Lasota)

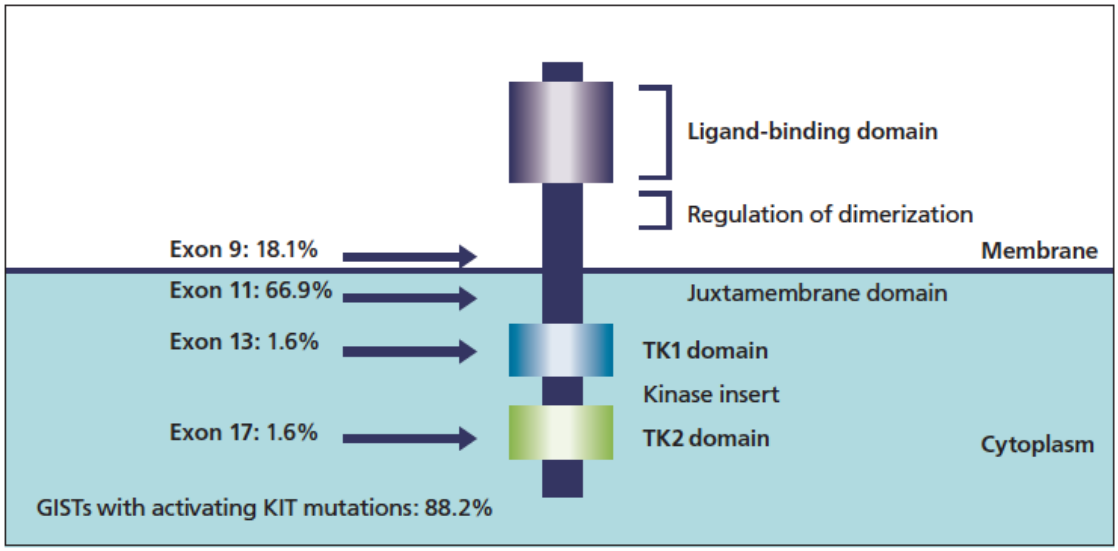
c. Mutational analysis

Overexpression of the KIT protein (CD117) is a defining feature of GIST [113]. Kit protein is a receptor tyrosine kinase product of the KIT gene. It has been identified as a proto-oncogene, localizing in humans to chromosome 4q11-q12 and encoding a 145 kilodalton transmembrane glycoprotein for a growth factor known as stem cell factor (SCF) or mast cell growth factor. Extracellular binding of SCF to the receptor results in the dimerization of adjacent KIT molecules with concomitant activation of the intracellular KIT kinase domain leading to activation of intracellular signaling cascades controlling cell proliferation, adhesion, and differentiation.

KIT is critical to the function of interstitial cells of Cajal and to normal gastrointestinal peristalsis, and also plays an important role in hematopoiesis, melanogenesis, and gametogensis[114] .

Activation of the KIT receptor tyrosine kinase is essential in the development of most GISTs[115]. This activation can involve different mutations within the c-kit gene as shown in Fig 9. These mutations (including deletions and point mutations) result in gain

of function, thus, KIT signaling is constitutively activated resulting in downstream phosphorylation in the signal transduction pathway, ultimately leading to increased cellular proliferation, resistance to apoptosis and defect in adhesion.



Abbreviations: GIST = gastrointestinal stromal tumour; TK = tyrosine kinase

Fig 9. GIST KIT mutations

The exact Kit mutation may be predictive of the risk of recurrence: deletion of the codons Trp 57 and Lys558 in exon 11 is associated with higher incidence of relapse and poorer clinical outcome, missense point mutations in exon 11 are associated with better prognosis only in gastric tumor localization, mutations in exon 9 are associated with GIST of the small bowel and are aggressive while mutations of the exon 13 are very rare (1%) and very aggressive [116] [117].

In a minority of cases, GISTs result from mutational activation of the closely related tyrosine kinase PDGF receptor a (PDGFRA) [115]. The majority of PDGFR-mutant GISTs are with gastric localization with epithelioid morphology, have weak or negative CD117 expression and are indolent tumors[108].

Approximately 15% of GISTs do not present any mutations in KIT or PDGFRA genes and are called wild type (WT) GISTs. Pediatric GISTs (female from adolescence to 40 years old) and Neurofibromatosis type 1 (NFS1) related GIST, belong to this group [118].

d. Treatment: the Imatinib Mesylate revolution

Surgery is the incipient therapy for patients with resectable primary disease and complete resection is achieved in approximately 70% of patients. Patients with advanced or inoperable disease have historically had limited treatment options, prior to 2001. Response to chemotherapy, such as doxorubicin, ifosfamide, temozolomide produced response rates of 5 to 10%. Also radiotherapy has little disease management role in GIST [119].

In 2001, Imatinib Mesylate was found to potently inhibit autophosphorylation of the tyrosine kinase receptor KIT and completely changed outcome for GISTs patients [120].

Imatinib (STI-571 (imatinib mesylate, Glivec, Gleevec™, Novartis Pharma, Basel, Switzerland) was found to readily inhibit ABL activity (Chronic myeloid leukemia-CML product of the t(9 ;22)). It was also noted that imatinib selectively inhibited KIT and PDGFRA kinase activity by competing for the adenosine triphosphate (ATP)–binding site of these receptors, found within the kinase domain. The FDA approved imatinib for treatment in GIST in 2002 and it remains a key first-line treatment in management of GIST today.

Imatinib is an effective treatment for unresectable and/or metastatic GISTs that affect the natural history (time to progression) of the disease. In addition, there is a consensus to treat all patients having 30% or higher risk of relapse, if their tumor carries a sensitive genotype. All patients with an intermediate risk (10-30%) should be evaluated on a case-by-case basis.

Response to imatinib varies greatly based on the underlying mutation type of the patient's tumor. Patients with KIT exon 11 mutation experience a significantly higher response rate, increased median and overall survival, and are at reduced risk of progression compared to patients with a KIT exon 9 mutation, or no KIT mutation at all. On the other end, patients harbouring exon 9 mutation, benefit of higher dose of Imatinb (800 mg/d) [121].

Evidence suggests that interrupted administration of imatinib does not prevent the development of resistance [122]. Furthermore, it has been demonstrated by Blay et al.

that interruption of treatment results in rapid disease progression in most patients with advanced GISTs [123]. Nevertheless, following the advent of imatinib treatment in GIST, resistance to the drug was shown to occur with the acquisition of secondary mutations in KIT. These missense mutations, occurred in KIT exons 13 and 17, are observed in addition to continue expression of the primary mutation, and significantly decrease the sensitivity of the KIT receptor to inhibition by imatinib, then impacting response [124].

Patients with WT GIST could benefit of other treatment in on-going studies. Paediatric GISTs are often associated with NF1 syndrome or with Carney Stratakis syndrome (often associated with the development of ganglioneuromas) and harbor WT Kit disease then, Imatinib does not appear to confer a significant benefit. Sunitinib, another drug tyrosine-kinase inhibitor, should be considered after failure of Imatinib treatment [118].

e. Role of the immune system in GIST

Two studies, respectively in 2008 and 2010 [125, 126] demonstrated the presence in GIST tumors of an immune infiltrate. In the first study, the immunohistochemistry (IHC) analysis in untreated c-KIT positive GIST, revealed that the most represented immune cells are DCs, but also T lymphocytes and monocytes. In the second IHC study, performed on primary or metastatic GIST mostly treated by Imatinib or Sunitinib, the authors demonstrated that GISTs are heavily infiltrated with macrophages and T lymphocytes. A relative high frequency of T cells expressed the nuclear factor FoxP3 (regulatory T cells) and the macrophages displayed the CD163 scavenger receptor, characteristic for anti-inflammatory M2 subtype, then both kinds of cells with an immune-suppressive phenotype. These two types of cells were close and in contact with tumoral cells. On the others hand, authors described HLA class I protein loss on tumor cells: 38% presented completely lack of expression of HLA –I A and 20% of HLA A-B-C taken together. This extensive loss of HLA class I molecules in GISTs suggested that tumor could induce a resistance to T-cells while it could be sensitive to NK cell lyses.

Unexpected long-term responses to IM in GIST patients with very low expression of KIT protein, suggested that IM could act indirectly on host cells outside of the tumor. Borg et al [127]selected several mouse tumors that do not response to IM *in vitro* but do *in vivo* in mouse model and demonstrated that tumors that are refractory to the

antiproliferative effects of IM in vitro (melanoma cell line) responded to IM in vivo in an NK cell–dependent manner. In fact, by inhibiting directly c-Kit on DCs, IM promotes an NK-DC cross talk that ultimately stimulates NK cells to produce IFNg. Moreover, in this study, authors assessed NK cell functions in 49 GIST patients demonstrating that NK cell functions were enhanced in 49% of Gleevec-treated GIST patients while only 20% of not-treated GISTs showed NK activation. Additionally, Gleevec-mediated NK cell activation correlated with clinical outcome because patients who displayed enhanced NK cell functions din not progressed and showed a significant better time to progression compared with patients with no NK cells activity.

Menard et al. confirmed this finding in a larger cohort of GIST patients (77 patients) treated with IM[128]. Moreover, the production of IFNγ by NK cells activated in the DC-NK cross talk promoted by IM was the only independent prognostic factor for progression-free survival, whereas the others known prognostic factors (c-Kit mutation status, diameter of the largest lesion, granulocyte count, IM dosing, and primary tumor site) were not predictive of response. These results indicated that the innate immune response is a major and independent predictor for progression-free survival in patients with advanced GIST receiving IM. Moreover, a comprehensive analysis of the GIST-associated NK cell phenotype was performed. This analysis showed that GIST patients, compared to healthy volunteers (HD), presented a down-regulation of the NKp30 receptor.

To validate this finding, mice bearing tumor lung metastasis (B16F10) were treated with the association of IM and IL-2 with great results based on an anti-tumor response involving NK1.1+ cells, tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), and IFNγ [129].

Moreover, a phase I clinical trial performed in Gustave Roussy Institut (IGR) from 2007 to 2009 in patients with refractory solid tumors, including 1 GIST, with IM associated with IL-2 and cyclophosphamide, demonstrates the activation of NK-cells with high expression of HLA-DR, TRAIL and CD56. The increase of this population correlated with an increase in overall survival (OS) and progression free survival (PFS)[130].

All these data underline the role of immunity in GIST control and, especially, the anti-tumor efficacy of IM *via* innate immune system activation.

To understand why some patients do not activate NK under IM treatment, Delahaye, Rusakiewicz et al, as mentioned in the previous chapter (see Supplemental Paper 1), performed an exhaustive study on NKp30 in GIST based on the previously observation of a down-regulation of this receptor in not treated GIST patients [128]. In this study is demonstrated that the alternative splicing of exon 4, which affects only the intracellular domain of NKp30, generates three membrane-bound proteins with distinct functions. The NKp30a and NKp30b isoforms induce a Th1-type cytokine secretion and NK degranulation (mostly NKp30a), while the NKp30c isoform induce the IL-10 secretion with an immune-tolerant response (decreased NKp30 dependent TNFa and CD107a release). The predominant expression on NK cells of one of these isoforms, correlate, in a cohort of 80 GIST patients with metastatic or recurrent disease under IM treatment, with OS: patients with a predominant NKp30a or NKp30b expression on peripheral NK cell, presented better OS compared to patients with predominant NKp30c expression. The NKp30 expression patterns are controlled by genetic factors, the NCR3*3790 SNP is associated with predominant expression of the NKp30c isoform.

The importance of the role of NK cells in GIST IM treated has been thwarted by Balachandran and colleagues [131] by the demonstration in a spontaneous GIST mouse model, that the depletion of CD4+T or NK lymphocytes did not affect tumor growth during IM treatment. Authors demonstrated that IM induces CD8+ lymphocyte activation and Treg apoptosis in the tumor. IM reduces Treg proliferation by inhibition of the Ido enzyme (indoleamine 2,3-dioxygenase) within the tumor, known to promote the development, stabilization and activation of Tregs. In parallel, the analysis of Ido protein in 13 freshly isolated GIST, showed a correlation between CD8+Teffector and Treg (linear correlation between Ido and Treg/CD8+ ratio).

A recent study from the same group underlines the negative role in progression free survival of neutrophils infiltrate compared to lymphocytes infiltrate, in untreated GISTs [132].

Taken together, all these studies demonstrate the important role of immunity in GIST development and the role of IM in modulating the immune cells. Which immune-actor is the protagonist in the control of disease (T lymphocytes or NK lymphocytes) is still an open question.

1.4 NEUROBLASTOMA

a. Characteristics of disease

Neuroblastoma (NB) originates from sympathetic tissue (neuroectodermic origin). It is the most represented extra-cranial cancer in children and 95% of NB patients are less than 5 years old.

NB clinical presentation and history is extremely variable since some localized or metastatic NB could regress spontaneously, others can be healing after chemotherapy and/or surgery treatment; other tumors cannot be cured even despite an aggressive treatment [133].

Epidemiology: NB is the third most common childhood cancer, after leukemia and brain tumors, and is the most common solid extracranial tumor in children, with an incidence of 8 children per million under the age of 15 are diagnosed with NB. It accounts for approximately 15 percent of all pediatric cancer fatalities.

Incidence rates are age-dependent, peaking during infancy and rapidly declining with older age. Children less than 5 years of age account for nearly 90 percent of all NB diagnoses in a given year. The median age at diagnosis is 17.3 months, and 40 percent of patients are diagnosed before one year of age. The incidence of NB is greater among white than black infants (ratio of 1.7 and 1.9 to 1 for males and females, respectively), but little if any racial difference is apparent among older children. NB is slightly more common among boys compared to girls[134].

No genetic predisposition factors have been identified in NB even if rare familial NB, association with Hirshprung disease, Ondine syndrome and PHOX2B gene anomalies, are described [135]. Other syndrome associations are with Beckwith-Wiedmann syndrome, NF1 and Di-George syndrome.

Symptoms: NB has been called the great mimicker because of its myriad clinical presentations related to the site of the primary tumor, metastatic disease, and its metabolic tumor by-products. NB presentation could be really heterogeneous mostly linked to the primary site of disease or to the metastasis. Due to the origin from primordial neural crest cells, which ultimately populate the sympathetic chain and the

adrenal medulla, 80% of primary NB occurs in the abdomen, with most of these occurring in the adrenal gland, 2% occurs in the cervical region, 12% in the chest and 5% in the pelvis. As a result, most children present with abdominal symptoms, such as fullness or distension, cervical or chest NB can present Claude Bernard Horner syndrome or superior vena cava syndrome, NB with cord compression (7-15%) present weakness, gait change, numbness, paresthesias, or incontinence and require urgent attention to reduce the risk of irreversible neurologic damage.

The most common finding upon physical examination for abdominal NB is a non-tender, firm, irregular abdominal mass that crosses the midline.

About 60% of NB presents a metastatic disease often involving bone marrow dissemination [136]. These patients present diffuse bone pain, fever, and lameness. Proptosis and periorbital ecchymosis are common in these high-risk patients (Hutchinson syndrome) and arise from retrobulbar metastasis. Extensive bone marrow metastasis may result in pancytopenia [137]. On rare occasions, children may have severe, watery diarrhea due to the secretion of vasoactive intestinal peptide by the tumor, or may have protein-losing enteropathy with intestinal lymphangiectasia .

Children with NB rarely present with paraneoplastic neurologic findings, including cerebellar ataxia or opsoclonus/myoclonus. Opsoclonus/myoclonus syndrome is frequently associated with persistent neurologic and cognitive deficits, including psychomotor retardation [138]. The opsoclonus/myoclonus syndrome appears to be caused by an immunologic mechanism that is not yet fully defined. Patients who present with this syndrome often have neuroblastomas with favorable biological features and are likely to survive, though tumor-related deaths have been reported.

In younger patients (<12 months), metastases are often localized in the liver. A particular presentation is represented by the Pepper syndrome (MS or IVs staging) presenting with metastatic disease limited to the skin, liver, and bone marrow. Pepper syndrome generally confers a better prognosis, as it is associated with spontaneous regression [139, 140]. Some infants with stage 4S neuroblastoma, however, die of massive hepatomegaly, respiratory failure, and overwhelming sepsis.

Diagnosis:

- **Biological tests:** NB can often be found by detecting catecholamines in the blood or urine. Sympathetic nerve cells normally release hormones called catecholamines, such as epinephrine (adrenaline) and norepinephrine, which enter the blood. Eventually the body breaks these down into metabolites, which are then passed out of the body in the urine. The 2 catecholamine metabolites most often measured in NB diagnosis and follow up are homovanillic acid (HVA) and vanillylmandelic acid (VMA). Only 5-10% of NB are not able to release HVA and VMA. Other non-specific blood biological tests routinely used are: NSE (Neuron Specific Enolase), ferritin and LDH (lactate dehydrogenase).
- **Imaging tests:** radiography, Ultrasound, Computed Tomography (CT), Magnetic resonance imaging (MRI), are usually utilized to detect and define the extension of disease. Metaiodobenzylguanidine (MIBG) with iodine-123 (123 I) MIBG is used to identify sites of metastasis. Tumors that contain sympathetic tissue, such as NB, ganglioneuroblastomas, ganglioneuromas, medullary thyroid carcinomas, pheochromocytomas, and carcinoids, take up MIBG. MIBG has also been used to follow up the response to treatment in NB patients. One of the drawbacks of using MIBG is that up to 30% of NBs may not take up MIBG, although 95% of NBs secrete catecholamines. Also, up to 50% of recurrent NBs do not take up MIBG even if they did so before therapy [141, 142].
- **Bone Marrow involvement:** bone marrow aspiration and/or biopsy are mandatory to identify a bone marrow involvement. Detection of typical pseudo-rosettas defines the NB infiltration [143].

b. Histopathological features

NB tumour cells are small round blue cells with hyperchromatic nuclei and a scant amount of cytoplasm. Neuritic processes and Homer-Wright pseudo rosettes are often seen also in bone marrow involvement. Other small round blue cell tumors, which must be differentiated from NB include Ewing's sarcoma, lymphoma, and rhabdomyosarcoma. Within one tumor specimen, different stages of differentiation

of neuroblastoma may be found, from undifferentiated NB to mature ganglioneuroma. The new International Neuroblastoma Pathology Classification (INPC) system (Table 1) has been developed from the initial Shimada classification of NB [144], taking into account the age of the patient, the mitotic-karyorhexis index (MKI), the amount of Schwann cells, and degree of cellular differentiation. The INPC system has been proven as a useful prognostic indicator in recent years.

Age	Pathology	Prognostic group
<1.5 years	Poorly differentiated or differentiating Low or intermediate MKI	Favourable
1.5 – 5 years	Differentiating Low MKI tumour	Favourable
<1.5 years	Undifferentiated High MKI	Unfavourable
1.5 – 5 years	Undifferentiated or poorly differentiated Intermediate or high MKI	Unfavourable
>5 years	All tumours	Unfavourable

Tab 1. Pronostic evaluation of NB according the INPC system (from Shimada et al 2001)

c. Biological features

A majority of NB tumors arise spontaneously through genetic and epigenetic changes in somatic cells (i.e. non-germ line cells). Sporadic neuroblastoma is usually associated with a multitude of large-scale genetic aberrations, such as gain or amplification of genetic material or hemizygous deletions. These DNA copy number changes are generally divided into numerical aberrations, affecting whole chromosomes, and segmental

changes, affecting only a part of the chromosome. However, except for the MYCN amplification, the driving genes of these regions are largely unknown.

NB cells frequently have cytogenetic signs of gene amplification, either as double minutes (DMs), i.e. small extra-chromosomal markers, or as homogeneously staining regions (HSRs) inserted into a chromosome. In 1983, these abnormal structures were shown to contain the MYCN gene, normally located at 2p24 [145-147]. This amplification, containing sometimes more than 100 copies of the MYCN gene, is present in 15-30% of NB tumors and is known to be a marker of poor prognosis. The discovery of MYCN proto-oncogene, has led to its development to become the most important biological prognostic factor in neuroblastoma [148, 149]. It is amplified in 5-10% of infants and in 20-30% of childhood and adolescent cases and it is strongly correlated to rapid disease progression and poor outcome in patients, regardless of age and stage.

Several studies suggested that NB could be considered as a genetic disease: 1) the observation that certain alleles of specific genes (FLJ22536, BARD1 and NBPF23) significantly increase the relative risk to develop NB [150] 2) the discovery of mutations in genes such as ALK (8% of tumors) or PHOX2B in rare familial cases [151] 3) a large number of recurrent genetic somatic alterations have been described in NB.

Numerical chromosome alterations are observed more frequently in tumors of younger children with localized disease and a good prognosis, whereas segmental chromosome alterations are found more frequently in tumors of older children with advanced stages of disease and a poorer outcome [152]. The overall copy number or ploidy of NB tumors has been found to be of prognostic importance, at least in infants, and is currently included as a prognostic marker in the treatment stratification of NB. Favorable tumors of lower stages are generally associated with a hyperdiploid or near-triploid karyotype while more aggressive NBs are near-diploid or near-tetraploid.

In addition to these aberrations, other reoccurring segmental DNA copy number alterations have been described at chromosome arms 1q, 2q, 4p, 9p, 14q and 19q. These chromosomal imbalances might harbor genes important for tumor initiation and progression. In this respect, chromosomal deletions could harbor putative tumor suppressor genes whereas chromosomal gains might indicate the presence of

oncogenes. Recent studies have permitted to define a role to segmental alterations in tumor progression [153, 154] and permitted to integrate the staging system with the genetic data.

d. Staging

Over the years, there have been several staging systems for NB, which have sometimes been used in parallel to each other. The first system was the Evans staging system (Table 2), which taking into account tumor site, lymph nodes involvement, distant metastases, bone marrow involvement and defined extension of disease and infant special stage (IVS). In an effort to make results comparable through the world, the International Neuroblastoma Staging System (INSS) was established in 1988 and subsequently revised in 1993 [155] taking into account tumor respectability (Table 3).

Nevertheless, as surgical approaches differ between one institution and another, a working group representing the major pediatric groups around the world met in 2005 to develop the International Neuroblastoma Risk Group (INRG) classification system, which takes in account radiological as well as molecular characteristics of the tumor [156] (Table 4)

Stage	Description
I	Tumour confined to the organ or structure of origin
II	Tumour extends beyond organ of origin but not crossing the midline. Ipsilateral lymph nodes may be involved.
III	Tumour extends beyond the midline. Bilateral lymph nodes may be involved.
IV	Remote disease involving skeleton, organs, soft tissues, or distant lymph nodes.
IVs	Patients who would otherwise be stage I or II but with remote disease confined only to the liver, skin, or bone marrow (without bone metastasis).

Table2. The Evans staging system

Stage	Description
1	Localised tumour with complete gross excision, ipsilateral lymph nodes negative for tumour.
2a	Localised tumour with incomplete gross excision, ipsilateral lymph nodes negative for tumour.
2b	Localised tumour with or without complete gross excision, ipsilateral lymph nodes positive for tumour, contralateral lymph nodes negative.
3	Unresectable unilateral tumour crossing the midline with or without regional lymph node involvement; or localised unilateral tumour with contralateral lymph node involvement; or midline tumour with bilateral extension by infiltration or by lymph node involvement.
4	Dissemination to distant lymph nodes, bone, bone marrow, liver, skin, or other organs.
4S	Localised primary tumour in infants younger than 1 year (stage I, IIA, or IIB) with dissemination limited to liver, skin, or bone marrow.

Table 3. The INSS staging system

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS				NA	No		C Very low
	< 18				Yes		Q High
				Amp			R High

Ploidy: diploid (DNA index = 1.0); hyperdiploid (DNA index > 1.0 and includes near-triploid and near-tetraploid tumors). Risk: very low risk (5-year EFS > 85%); low risk (5-year EFS > 75% to <= 85%); intermediate risk (5-year EFS >= 50% to <= 75%); high risk (5-year EFS < 50%). Histology: GN, ganglioneuroma; GNB, ganglioneuroblastoma. *MYCN*: Amp, amplified; NA, not amplified. Stage: L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age; EFS, event-free survival.

Table 4. The International Neuroblastoma Risk Group consensus pre-treatment classification schema

e. Treatment

NB therapy is stratified according to the risk grouping described above (Fig 10).

Variable	Category			
	Low risk	Intermediate risk	High risk	Stage 4S tumours
Pattern of disease	Localized tumour	Localized tumour with locoregional lymph-node extension; metastases to bone marrow and bone in infants	Metastases to bone marrow and bone (except infants)	Metastases to liver and skin (with minimal bone marrow involvement) in infants
Tumour genomics	Whole-chromosome gains	Whole-chromosome gains	Segmental chromosomal aberrations	Whole-chromosome gains
Treatment	Surgery	Moderate-intensity chemotherapy; Surgery	Multimodal therapy	Supportive care
Survival rate (%)	>98	90 to 95	40 to 50	>90

Fig 10. Neuroblastoma staging and co respective treatment and survival rate

For low risk patient's tumor surgical resection may be curative [157-159] also when surgery is incomplete and for some tumors even observation alone could be the best

approach. For metastatic tumors, tumor resection has been debated. However, as local relapse is common in patients with metastatic disease, surgical removal of the primary tumor is recommended in most high-risk treatment protocols.

Children with high-risk disease have a poor prognosis (Fig 11) but it has been shown that they need multimodal therapy with intensive induction chemotherapy [160] followed by an attempt to radical surgery and irradiation of the primary tumor to achieve local control. High dose chemotherapy with stem cell rescue has a significant advantageous effect in order to eliminate residual disease after induction [161] usually preceding the local treatment and maintenance therapy with retinoic acid [162]. There are many chemotherapeutic agents used in NB treatment today, such as alkylating drugs (cyclophosphamide, busulphan, melphalan), vinca-alkaloids (vincristine), antracyclines (doxorubicin), and platinum analogues (cis-platinum, carboplatinum). Further options include agents such as topotecan, irinotecan, and temozolomide that are currently being tested in phase II-III clinical trials [163]. Initial therapy (induction) in the European protocol HRNBL-I consists of intensive therapy with several agents, such as the COJEC protocol (cisplatin (C), vincristine (O), carboplatin (J), etoposide (E), and cyclophosphamide (C). Intensive research suggested some new strategies that were later added to the current treatment protocols. The most accomplished treatments involve 13-cis retinoic acid administration to help differentiate the highly proliferating tumour cells and immunotherapy which consists of a monoclonal antibody to a disialoganglioside GD2 located on the surface of the tumour cells added with interleukin-2 and/or a granulocyte-macrophage colony-stimulating factor [164].

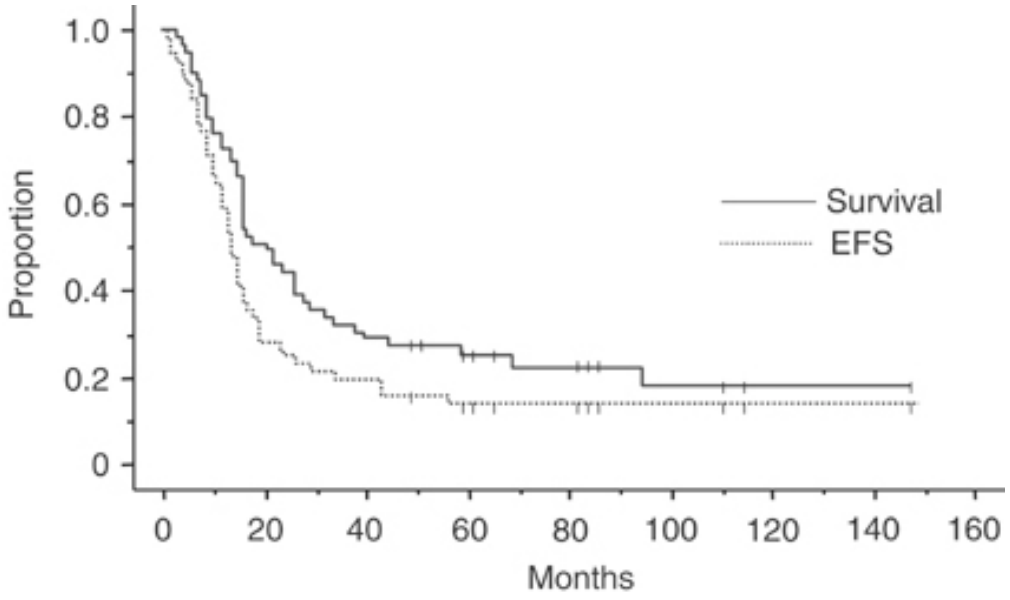


Fig 11. OS (black line) and EFS (dotted line) for metastatic NB (adapted from Luksch et al Br J of Cancer 2005)

f. New drugs

Experimental therapies mainly target biological anomalies in NB. Down regulation of MYCN expression using RNA interference (RNAi) in MYCN-amplified cell lines resulted in increased apoptosis and differentiation of the cells [165]. In addition, treatment of MYCN-amplified neuroblastoma cell lines with an Aurora kinase inhibitor, CCT137690, can also decrease MYCN protein expression and therefore inhibition of cell proliferation [166]. This effect can be explained by the fact that the Aurora kinase has been proven to stabilize the MYCN protein in neuroblastoma cells. A second relevant biomarker and interesting therapeutic target that has recently emerged is the receptor tyrosine kinase ALK (Fig 12). As previously discussed, the ALK gene is mutated in a subset of neuroblastoma tumours thereby enhancing tumour proliferation. Intensive drug screens indicated two ALK inhibitors, TAE684 209 and PF-02341066 or crizotinib [151, 167], which are able to reduce proliferative potential of neuroblastoma cells containing these ALK mutations. More recently, new inhibitors have also been introduced. Recently, histon-deacetylase (HDAC) inhibitors also became of increasing importance as anti-cancer treatment strategy. For neuroblastoma, Witt and colleagues reported that treatment of neuroblastoma cell lines with HDAC inhibitors induces a wide range of distinct phenotypes such as cell cycle arrest, differentiation and apoptosis [168]. Also, as angiogenesis is one of the main characteristics of neoplasm formation, anti-angiogenesis

agents define a putative new drug treatment for neuroblastoma. An example of such an agent is fenretinide which was shown to induce programmed cell death of human neuroblastoma cells [169].

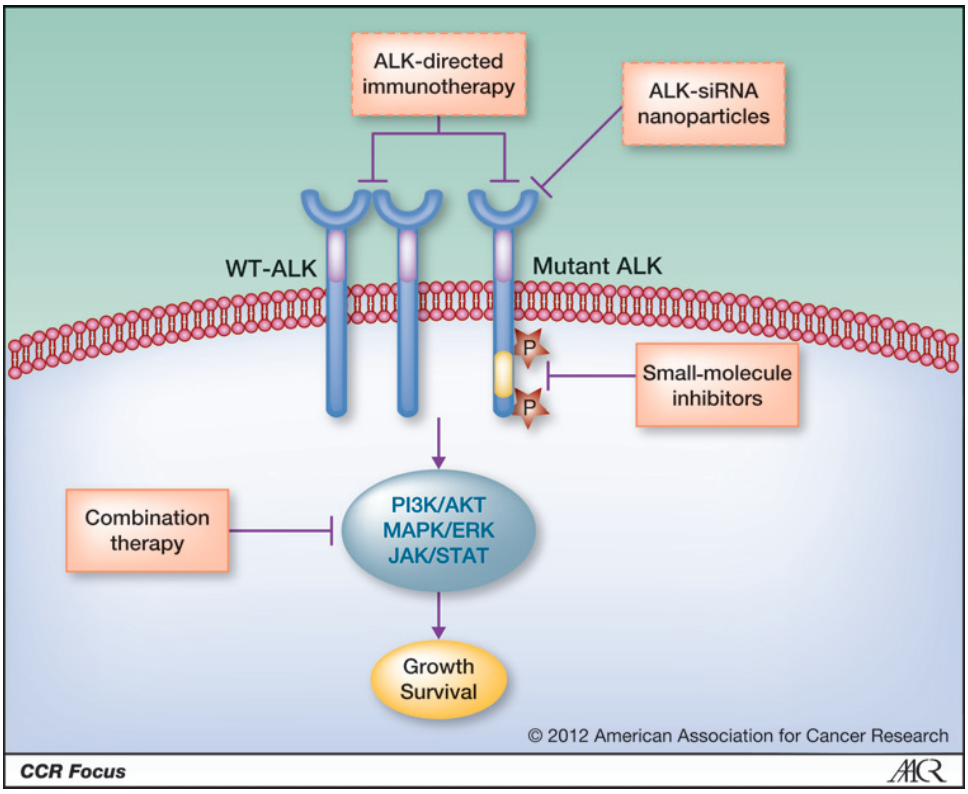


Fig 12. Targeting ALK pathway (adapted from Matthay et al 2012 Clin Cancer Res)

g. Neuroblastoma and Immune system

Since 1968 evidences for immune mechanisms in NB patients were provided:

1. The demonstration that lymphocyte suspensions from nine NB patients inhibited autologous and allogenic colony formations by neuroblastoma cells. Moreover plasma samples obtained from the same patients in the presence of complement inhibited neuroblastoma colony formation to a significant degree. [170]
2. Evidences of lymphocyte infiltrations within the NB tissue and correlation with patient age and survival [171]
3. Evidences of spontaneous tumor regressions [172] supported by Squire et al who found that stage IVS/MS tumors had a higher MHC I expression, supporting the hypothesis that spontaneous regression might be immunological mediated .

Over the time, one of the things that became of evidence, is that NB interacts with its microenvironment and, particularly, with immune cells. The immune system can kill cancer cells but can also be commandeered by tumor cells to promote tumor growth and suppress anti-tumor immunity. This concept has been well explained by Robert C Seeger and defined “Yin and Yang” interaction of the immune system with cancer cells [173]: several actors of the immune system play as a “balance” in NB with pro-tumor and anti-tumor functions.

Immune evasion of NB

NB use different mechanisms to evade recognition by effector cells of the immune system including

1) Immune tumor microenvironment in NB promotes tumor growth. Microarray analysis in HR-NB revealed high expression of genes related to B-lymphocytes, macrophages and inflammation including IL-6, IL-6R, IL-10 and TGFb. TAMs secreting IL-6 (tumor associated macrophages) also have been described in HR NB. [174, 175]
2) NB express immunosuppressive cytokines like TGF-b and/or IL-10 which may prevent activation and expansion of tumor-infiltrating lymphocytes [176]

3) NB express B7-H3, an immunosuppressive molecule which confers protection of tumor cells from NK cell-mediated lyses [177]
4) NB down regulate co-stimulatory molecules such as CD40, CD80 or CD86 [178]
5) NB cells produce high levels of macrophage migration inhibitory factor (MIF) which may cause activation induced T-cell death and contribute to NB progression and induce VEGF production by NB cells. Furthermore NB cells produce also the pro-angiogenic chemokine IL-8 which can blocks TRAIL-mediated apoptosis [179].
6) Norepinephrine, the neurotransmitter produced by NB cells, is a potent inhibitor of NK cell activity (Lang K Immunology Letters 2003).
7) NB cells often are devoid of MHC I antigens which protects NB from T cell mediated immune surveillance. On the other hand, the lack of MHC class I expression makes NB cells an ideal target for NK cells.[180] MHC I expression can be enhanced by chemotherapy treatment. In NB, high n-Myc levels correlate with low HLA class I levels, suggesting an involvement on N-Myc regulation in HLA expression.
8) NB cells may down modulate their expression of NKG2D ligands such as MIC and ULBP, providing them with a potential to escape death mediated by NKG2D expressing effector cells. Furthermore, it was shown that sera from NB patients contained soluble MICA, which could impair NKG2D expression on cytotoxic cells and consequently their cytotoxic potential. An other soluble factor with immunosuppressive role found in NB patients is soluble HLA-G produced by monocytes in contact with NB tumor, which was found higher in patients at diagnosis and correlated with relapse [92].

9) In vivo experiment showed that Treg depletion enhanced vaccine-induced immunity in mice treated by Neuro 2A/IL-21 vaccine [181].

Susceptibility of NB to immune cells mediated control

1) **Cytotoxic T lymphocytes:** NB cells express antigens that may be recognized by CTLs, among these there is N-Myc, germline antigens such MAGE and NY-ESO, PRAME (known as melanoma antigen), survivin, GD2 synthase and telomerase. The lack of MHC I described in the previous paragraph, explain the escape of NB from

CTL recognition [182]. Interestingly, the expression of MHC class I was significantly up regulated upon IFNg treatment in a panel of NB cell lines [183]. Accordingly, the expression of MHC class I molecules on NB cells stimulated with IFNg appears adequate for CTL recognition and lysis. Nevertheless, CTL based clinical trials have not progressed beyond pilot and phase I studies.

2) **Natural Killer lymphocytes:** lack of MHC class I expression may render NB cells an excellent target for NK-mediated cytotoxicity. Several studies have demonstrated NB susceptibility to NK cytotoxicity: Venstrom and colleagues [184] demonstrated that killer immunoglobulin-like receptor (KIR) and HLA gene polymorphisms, which interact to govern NK cell function, correlated with disease progression and survival in patients with high-risk disease treated with autologous hematopoietic stem cell transplant (AHSCT). Those with a "missing ligand" KIR-HLA compound genotype had a 46% lower risk of death at three years after AHSCT compared to patients who possessed all ligands for inhibitory KIR. Delgado and colleagues, in another study, analyzed the response to antiGD2 immunocytokine hu14.18/IL-2treatment in relapsed or progressive NB, and demonstrated that patients with a KIR-HLA ligand mismatch presented a significantly better response [185].

The known activating receptors involved in NK cell–mediated recognition and/or cytotoxicity are: NKG2D by the evidence of NKG2D ligands on NB cells[92], the natural cytotoxicity receptors (NCRs) even if their ligands on NB cells are unknown, and DNAM-1 by the in vitro studies of involvement in NK cell cytotoxicity against NB cells[186]. Most of the cytolytic activity was confined to the NKp46 and NKp30/NKp44 bright subsets, suggesting that down-regulation of NCR ligands on the NB cells can affect their killing by NK cells. The role of NK cells in anti GD2 immunotherapy will be discuss later.

3) NKT cells: Vα24-invariant (type I) natural killer T cells (NKTs) react to self- and microbial-derived glycolipids presented by the monomorphic HLA class-I-like molecule CD1d. They express an invariant TCR α-chain, Vα24-Jα18 which is preferentially paired with Vβ11[23]. NBs do not express CD1d, however, NKT cells, if stimulated, secrete IL-2, which activates NK cells, and so indirectly NKT cells can

mediate cytotoxicity against neuroblastoma cells [187]. NKT infiltration in NB is correlated with MCP-1/CCL2 expression by tumor, which is in turn inversely correlated with N-Myc amplification. In fact N-Myc can impact the CCL2 expression to limit NKT infiltration. NKT cells can kill monocytes pulsed with tumor lysates then NKT lymphocytes mediate antitumor activity via killing of TAMs [174].

h. Immunotherapy of Neuroblastoma

Due to the sensitivity to NK lysis, several approach in NB immune treatments have been evaluated. Here it will be shown the immune treatment already experimented in clinical trials.

First, IL-2 treatment to induce amplification of the NK compartment was used in numerous trials, IL-2 treatment was well tolerated and induced both the proliferation of circulating NK and T cells as well as the activity of NK cells. However these clinical trials did not demonstrated major clinical responses if not associated with other treatments [160, 188]. Instead, administrating IL-2 as an adjuvant to anti GD2 therapy or direct delivery to tumor microenvironment by fusing it with anti GD2 antibody could be more effective. Several studies have shown the potency of IL-2 to activate NK cells resulting in increased antibody-dependent cell mediated cytotoxicity (ADCC) mediated by anti GD2 antibodies [189-191].

Immunization of NB patients with IL-2 transduced autologous tumor cells achieved some clinical responses, which correlated with a detectable anti-tumor CTL activity. There are no clinical studies that demonstrate GD2-specific CTLs recognizing tumor in NB patients. A novel strategy is to immunize with DNA encoding a GD2-mimicking peptide, which induced both a humoral and an NK-mediated immune reponse [192].

Targeting the disialoganglioside GD2, highly expressed by NB cells has been an enormous challenge in HR NB treatment. Immunotherapeutic targeting of GD2 aims at directing the hosts' Fc-receptor-positive NK cells, granulocytes and macrophages, against the tumor cells. It has been shown that anti-GD2 antibodies induce killing of NB cells by both ADCC and complement dependent cellular cytotoxicity (CDC) [193] (Fig). Three variants of monoclonal antibodies are developed for clinical use: the

murine IgG3 mAb 3F8, the murine IgG2a mAb 14G.2a and the human-murine chimeric mAb ch14.18. Passive immunotherapy using any of these three antibodies has demonstrated complete remissions and prolonged EFS in phase I/II trials [194]. To further stimulate the immune system, combinations of anti-GD2 therapy with cytokines administration (IL-2 and GM-CSF in USA or IL-2 in the European protocol) have been employed with a remarkably outcome [164].

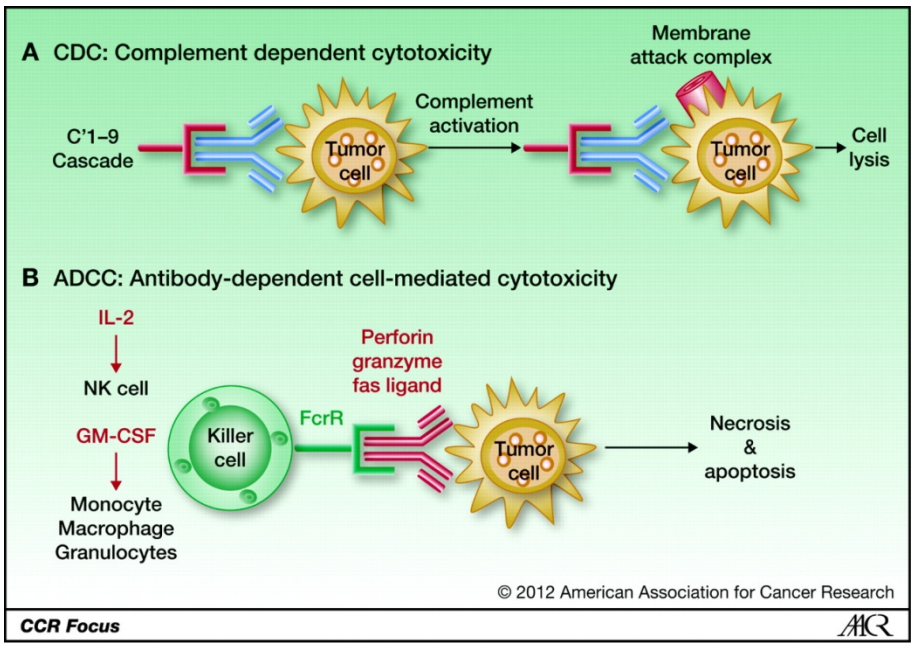


Fig 13. Mechanism of GD2 antibody-targeted destruction of NB (from Matthay et al Clin Can Res 2012)

Although ch14.18 in combination with IL-2 and GM-CSF has been shown to improve the outcome of HR-NB, the treatment is associated with significant toxicities, especially in cycles containing IL-2. Furthermore, the exact contribution of cytokines in vivo remains unclear. To address these issues, the Society of Pediatric Oncology European Neuroblastoma Network is conducting a study that randomizes patients with high-risk NB to ch14.18 alone or in combination with infusion IL-2 to ameliorate toxicities (phase III HRNBL1 protocol).

Second generation of GD2 targeted immunotherapy are now developing:

- Hu14.18-IL2 that is a fusion protein of humanized anti-GD2 antibody (hu14.18) and IL-2 [195]

- Hu14.18K332A is a humanized ch14.18 with a mutation to alanine at lysine 322 that limits its ability to fix complement and thereby reduces the pain associated with ch14.18 while retaining its ADCC capabilities [196]
- Monoclonal antibody 1A7 is an anti-idiotype antibody that is directed against a murine anti-GD2, 14G2a [197]. Its use in a clinical trial in HR-NB who achieved first or subsequent responses as a vaccine was well tolerated and induced an active biological activity anti-GD2 (CDC and ADCC activities), it could then be useful for controlling minimal residual disease (Yu et al unpublished data).

One of the most promising therapies for treatment of high-risk NB involves the use of autologous human T lymphocytes genetically modified to express chimeric antigen receptors (CARs) that recognize tumor antigens and provide activating signal to the T cells. These receptors use a single chain fraction variable (scFv) antibody-derived motif for recognizing a cell surface antigen; therefore the recognition is independent of antigen processing or MHC class I-restricted presentation. Currently, these CAR-T cells have been used to mediate tumor regression in patients with NB, and offer another testable strategy for GD2-directed immunotherapy as well as for any specific NB antigen [198].

2. RESEARCH OBJECTIVES

The overall aim of this thesis was to characterize the immunological actors and protagonists, which play a prognostic role in two cancer diseases known to be NK sensitive: Neuroblastoma and Gastrointestinal Stromal Tumors.

Both advanced GIST and High Risk NB (HR-NB), present an unfavorable outcome despite the goal treatment Imatinib (IM) in GIST and the very aggressive treatment utilized in HR-NB.

The introduction of IM had a remarkable impact in the management of GISTs, but the different clinical trials and the collaborative research studies have demonstrated that we can not consider GIST as a single entity because different molecular subsets of GIST have very different prognosis in both advanced and localized phase.

In the same way, metastatic NB is another entity compared to localized NB with different clinical characteristic and sensitivity to treatments. Likewise response to treatment and clinical evolution among HR-NBs with the same biological features could be extremely different.

Taken together, these observations lead us to consider that changing the “point of view” could get round the problem and may help us to understand other mechanisms that are also responsible of the different tumor behaviors. In this thesis we will focus on the “immunological point of view”.

NK cell have been demonstrated to play an important role for NB disease and previous immunomonitoring studies carried out in GIST revealed a defect in NK cells function with a particular down-regulation of NKp30 receptor in the peripheral blood of GIST patient that predict patient clinical outcome [127, 128]. These observations prompt us to investigate the immune infiltrate in primary localized GIST tumor and evaluate their prognosis factor. In parallel, we performed an immunomonitoring of NB patients to characterized the lymphocytes compartment, evaluate NKp30 protein expression and evaluate the isoform transcriptional profile. We then correlated these parameters with clinical response.

These immunological scores both in GIST and in NB, could permit a better understanding of the immune cell population capable of maintain the tumor cells control. This information will allow us to better evaluate the appropriate therapy that would consent to the immune system to control the tumor outgrowth.

In **Paper 1,** we performed an in depth analysis of the immune infiltrate in GIST tumor samples at diagnosis focusing on NK, Treg, and T lymphocytes. The aim of this work was to characterize the prognostic value of tumor-infiltrating lymphocytes GIST and to investigate the impact of IM on both tumor cells and immune infiltrate.

The second aim of this thesis was to characterize the immune infiltrate in bone marrow infiltrate compared to peripheral blood samples in NB patients at the time of diagnosis. According with the literature evidences on NK role in NB disease, we primarily focus on NK characterization. Then we direct the research on the NKp30 receptor and, based on our previous study on impact of the NKp30 isoforms in GIST tumor, we explored the NKp30 isoforms expression in NB patients and we correlated the results with the clinical evolution of HR-NB. Accordingly, we analyzed the expression of the NKp30 ligand, B7-H6, on NB cells, and, more generally, in NB patients. The results of this work are presented in **Paper 2.**

2.1 Paper 1

Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors

In this paper we wanted define the immune infiltrate in GIST. We already known that cancer immuno-surveillance and -editing relies on the presence of effector/memory tumor infiltrating T cells of a Th1 profile. The previous researches focused on NK role in controlling GIST disease have led us to examine the NK infiltrate in primary GIST.

We first demonstrated that, contrary to soft tissue sarcomas, localized GIST are enriched in activated NK cells in fibrous trabeculae at diagnosis. Moreover, after c-kit tyrosine kinase inhibitor imatinib mesylate treatment, NK cells accumulate inside tumor nests (NK TILs). We next phenotyped the NK TILs and we showed that they harbour a homogeneous phenotype ($CD56^{bright}$ $CXCR3^{+}$ $CD69^{+}$CD16-KIR- NKG2A-) and secrete IFNg/TNFa in tumor beds.

Interestingly, upon imatinib therapy, NK cells move to the tumor nest, while the number of infiltrating Foxp3+ lymphocytes decrease. To better understand this phenomenon, we performed an analysis of the MHC class I expression on GIST tumors before and after treatment (coupled paraffin embedded samples): after IM treatment 40% of GIST become partially negative to MHC class I, therefore NK cells can migrate in these areas while CD3+T cells are preferentially located in MHC class I positive tumor nests. Indeed GIST become suitable NK cell targets.

Importantly, NK and CD3+ T cell infiltrates at diagnosis in a cohort of 53 localized GIST were correlated with a better prognosis (reduced relapse rate). Multivariate analysis revealed that NK and CD3+ T cell infiltrations are independent prognostic factors for the progression free survival (PFS). On the contrary, low NK and or CD3+ TIL numbers markedly predict relapse in intermediate risk GIST, prompting the use of this biomarker for the clinical management of GIST.

Moreover these data prompt us to evaluate new therapeutic approaches to combine to target treatment in order to strengthen the NK and T lymphocytes cytotoxicity against tumor.

2.2 Paper 2

NKp30 isoforms dictate the prognosis of neuroblastoma

Neuroblastoma (NB) is a childhood malignancy that originates from the embryonic nervous system. Despite substantial advances in the cure of childhood cancer, NB with aggressive features has remained lethal for 60-70% of patients. NB affects only ~1.5 in 100.000 children, but it accounts for 15% of cancer deaths in this age group. Fatal relapses after current multimodality treatment regimens are attributed to dormant and chemoresistant tumor cells that persist throughout therapy (minimal residual disease, MRD). To improve cure rates, non-cross-resistant therapies are needed that efficiently eradicate MRD. Most of the High Risk NB (HR-NB) patients are older than 18 months at the time of diagnosis. The most common genetic lesion in NB is the amplification of MYCN, the incidence of MYCN amplification in NB population is around 30%.

Recently, the benefits of a maintenance treatment with immunotherapy combining IL-2, GM-CSF and anti-GD2 have been demonstrated in a randomized study supporting immunological based strategies [199].

Tumor lysis induced by NK cells relies on the commitment of Natural Cytotoxic Receptors (NCR: NKp44, NKp46, NKp30, NKp80 and NKGD2 and DNAM1) expressed on NK cell surface. Interestingly, tumor can shed NCR ligands in order to escape from immune system activation, these observations suggest that these ligands could be very effective for the recognition of tumor cells by innate immunity. Our previous study [65] demonstrated that alternative splicing of the gene NCR3/NKp30 almost exclusively expressed by NK cells, leads to formation of three isoforms. Functionally these isoforms regulate NK cells functions toward IL-10 secretion or IFNg/TNFα secretion and cytotoxicity. The 3 isoforms differ only in the intracellular domain while they present the same extracellular domain with no differences in the interaction with the known ligands (eg B7-H6). Study in patients bearing GastroIntestinal Sarcoma Tumors (GIST), GIST being highly sensitive to NK cells as NB, demonstrated the prognostic value of the expression profile of these three isoforms in terms of overall survival in a large cohort of patients treated with imatinib mesylate.

In this study we wanted to evaluate the NK characteristics at the time of diagnosis both in peripheral blood and in bone marrow in a cohort of localized and metastatic NB. We showed that metastatic NB presented higher NK cell infiltrate with prevalent NK^{bright} phenotype. These 2 characteristics were also associated with minor response to chemotherapy induction treatment in HR-NB.

Moreover the analysis of the NK receptors showed that NKp30 is down regulated in infiltrated bone marrow (compared to peripheral blood but also not-infiltrated bone marrow, not shown) that evokes an engagement of the same receptor.

In a second time we analyzed the expression of the NKp30 ligand, B7-H6, on the neuroblasts and we found that infiltrating bone marrow neuroblasts are mostly positive for B7-H6 like all the NB cell lines tested. Interestingly, the soluble B7-H6 was associated with the extension of the disease and with minor response to induction chemotherapy in HR-NB.

In vitro experiment demonstrated that the B7-H6 expression on NB cell lines could be modulated by several cytokines: while IFNγ induces a down regulation of the B7-H6 mRNA, IL-10 induces an upregulation. We demonstrated that monocytes are implicated in the NKp30-B7-H6 cross-talk *via* TNFα and IL-10 production.

We then addressed the question if NKp30 isoforms are involved in the NB natural history. The analysis performed on two independent cohorts of HR-NB with minimal residual disease after induction treatment, showed the protective role of the pro inflammatory NKp30 B isoform despite the immunosuppressive one (NKp30C) in predicting progression free survival (PFS). Moreover NKp30 isoforms could better predict PFS in the most 'problematic' HR-NB: children older than 18 months and without amplification oncogene n-Myc.

To our knowledge this is the first demonstration of the host role in the natural history of the NB disease. This prompts us to enhance the immunological NK responses and to prevent the immunosuppressive effects in the NKp30c profile patients

3. CHAPTER 3

DISCUSSION AND FUTURE PERSPECTIVES

The ultimate goal for cancer treatment would be to reach a state of personalized medicine, in which molecular, immunological, microenvironment characteristics of the tumor provides a basis for the selection of the most suitable therapy for each patient. In reality however, this is not unproblematic. Not only it's mandatory to have informative and reliable diagnostic methods that are capable of distinguishing between the relevant predefined tumor subgroups, but also to have a good understanding of the biological mechanisms characterizing the disease, and last but not least, tumor and host specific strategies to deal with the disease once it has been characterized.

As discussed in chapter 2, the effector functions of NK cells are regulated by the complex interaction of activating and inhibitory receptors. This tightly balanced regulation of NK cell activity can determine a status that favors the activating signals enhancing NK cell anti-tumor activity. This setting constitutes an attractive approach in cellular immunotherapy to treat cancer. To optimize this strategy an in-depth knowledge of the interaction between these NK cells and the tumor cells is required. In fact both cellular interactions and the local environment in which the NK cells resides in, may influence their cytotoxic functions.

The results presented in this thesis show that immune infiltrates and, in particular, NK lymphocytes actively contribute in tumor growth control both in GIST and in NB. These two tumors are different in embryonic origin, histological and clinic characteristics and natural history, and they can rarely share the expression of neuronal antigen like NCAM/CD56 [200]. Nevertheless, the common characteristic of NB and GIST tumors is the NK cells lysis sensitivity.

In our first paper we have demonstrated that TILs in GIST are particularly enriched in activated $CD56^{bright}$ NK cells localized in the tumor borders. NK sensitivity could be enhanced by IM via a progressive loss of MHC class I by the tumor cells and an increase of NK cells infiltration in the core of the tumor. Mechanisms underling the NK and T lymphocyte migration (probably du to a chemokines gradient) have to be elucidated. We demonstrated, for the first time, that PFS in localized GIST is controlled by both NK

and T lymphocytes immunosurveillance. Indeed NK and T lymphocytes infiltration are independent prognostic factors of disease progression in localized GIST.

Also in NB, $CD56^{bright}$ NK and Treg lymphocytes have a particular behavior; in fact they are more represented in metastatic patients (PB and BM) than in localized NB. Interestingly $CD56^{bright}$ NK cell levels could predict tumor sensitivity to induction chemotherapy.

Similarly to GIST, infiltrated NB-BMs present down regulation of the activating NKp30 receptor. As we demonstrated, this is due to the NKp30-B7-H6 cross-talk on NB cells and, moreover, to the immune escape mechanism induced by the soluble B7-H6 protein. Further research has to evaluate the role of B7-H6, sB7H6 and TGFβ secreted by NB cells or Tregs [201] [176] in the down-modulation of NKp30 on peripheral and BM infiltrated NK cells. B7-H6 and, especially, s-B7-H6 mechanisms of shedding and activation of the NKp30 receptor are currently investigated. Probably polymorphisms in the B7-H6 gene are responsible of the heterogeneity of the B7-H6/NKp30 cross talk.

Another common characteristic between GIST and NB is the impact of the NKp30 transcriptional profile, due to the alternative splicing of its intracellular domain leading to three major NKp30 isoforms with differential effector functions, on disease outcome. Indeed the preferential NKp30c isoform transcription leads to an unbalanced between IL-10/TNFα and impact the disease event-free survival. To our knowledge, this is the first demonstration of the host feature impact on the course of neuroblastoma disease.

In our research we confirmed that NKp30 profile is stable during time, despite chemotherapy treatment, and it is not modified in different organs (we tested for GIST peripheral blood, spleen, lymph nodes and tumors, for NB peripheral blood and bone marrow).

We have further analyzed NCR3/NKp30 expression in two non-tumoral diseases, Human immunodeficiency virus (HIV) and Primary Sjögren‘s syndrome (pSS) (supplemental article 2 and supplemental article 3) and found no impact of the NKp30 isoforms profile on outcome or evolution of disease.

In pSS, the presence of a genetic polymorphism of the NCR3/NKp30 gene has been associated with reduced gene transcription and function as well as protection to pSS compared to a matched control cohort, suggesting an inflammatory NKp30 related status in pSS development (Supp art. 3).

In HIV disease, NKp30 protein has been found down regulated in $CD56^{dim}$ and $CD56^{neg}$ NK subpopulations, but NKp30 isoforms do not impact the disease progression in a large therapy-naive HIV cohort (Supp art. 2).

On the contrary, in NB we have demonstrated that a high ratio between NKp30b/NKp30c isoforms impacts the TNFα/IL-10 cytokine balance, which in turn, predicts long-term survival (10 years) in two independent cohorts of HR-NB in complete remission of metastasis after induction treatment.

In conclusion, the results presented in this thesis increase our understanding of the immunogenicity of NB and GIST and their interaction with the immune system. As discussed earlier, the tumor microenvironment has a potent regulatory effect on the infiltrating immune cells. So the first line of action should be breaking the induced immune tolerance (like for example with Imatinib, which reduce Ido concentration). On another hand, we can intervene by potentiating the immune cells so that they conserve their effector phenotype (IL-2, IL-15, Roferon are currently associated in several clinical trials). Finally a combination of both strategies according to the type of cancer is very attractive, but remains to be tested.

The role of the NKp30 isoforms highlighted in this thesis constitutes an instrumental tool to new therapeutic options, particularly, changing the NKp30/NCR3 profile in patients with high expression of the NKp30c isoform in HR-NB during maintenance treatment or in HR-GIST combined to IM treatment.

As discussed before a single nucleotide polymorphisms (SNPs) NCR3-3790, causes a preferential expression of NKp30c in about 50% of patients with an NKp30c profile. Our unpublished/not shown data suggest that histone deacetylase (HDAC) inhibitors, like Vorinostat (SAHA) or Compound2 (Cp2), or demethylating agents like 5-azacytidine

(azacitidine) and 5-azadeoxycytidine (decitabine), are promising drugs capable of switching the NKp30 profile in vitro.

Despite these data, recent researches [202] demonstrated that HDAC inhibitors are able to induce down regulation of B7-H6 in *in vitro* model, reducing NKp30 dependent tumor cell recognition by NK cells. Moreover due to the HDACs involvement in controlling MYCN function, HDACs inhibitors are currently tested in phase I studies in NB[168].

Another treatment option in patients bearing the NKp30c isoform could be based on the neutralization of IL-10, which is known to exert direct growth inhibitory effects on tumor cells *in vitro* and *in vivo*. Antibodies anti-IL10 are currently used in different clinical trials with contrasting results. Another strategy might consist in the generation of bispecific antibodies targeting GD2 and NKp30, mostly in ΔBC-high patients to enhance the anti-tumoral NK activity.

Moreover, as we demonstrated in *in vitro* study, IFNγ, mostly secreted by NK in ΔBC-high patients, up-regulates B7-H1 expression on NB cells; then it could be of interest to evaluate an alternative strategy with the association of anti PD-1/PDL-1 treatment.

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RESEARCH APPOINTMENTS (Oral presentation)

- June 2012 ANR meeting TORONTO **"**Alternatively spliced NKp30 isoforms affect outcome in patients with metastatic neuroblastoma and minimal residual disease after induction chemotherapy", French Embassy Award.
- October 2011 SIOPEL meeting, Paris "Liver tumor in adolescent and young adults"
- November 2011 London Neuroblastoma Meeting "NKp30 polymorphisms in Neuroblastoma"
- October 2009 "Relapses in hepatoblastoma patients: clinical characteristics and outcome - experience of the international childhood liver tumor strategy" International Society of Pediatric Oncology, SIOP annual meeting, Sao Paulo, Brazil.
- March 2009"Analysis of hepatoblastoma relapses » Munich Reunion Group International SIOPEL-Munich, Germany
- June 2008 "Procalcitonin as a predictor of unfavorable outcome in neutropenic pediatric patients presenting with fever of unknown origin » Société Française de Pédiatrie(SFCE), Nantes/France
- September 2006 « Evidence of bipotential stem cells in hepatoblastoma »: 38th Congress of the International Society of Paediatric Oncology (SIOP), Gèneve, Suisse.
- December 2005 «Hepatoblastoma: molecular and immunohistochemical study»: Molecular Biology meeting- Institut Pasteur, Fontainebleau/France.

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Supplemental papers

1- Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors
2- Analysis of NKp30/NCR3 isoforms in untreated HIV-1-infected patients from the ANRS SEROCO cohort
3- NCR3/NKp30 Contributes to Pathogenesis in Primary Sjögren's Syndrome

nature medicine

Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors

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The natural killer (NK) cell receptor NKp30 is involved in the recognition of tumor and dendritic cells (DCs). Here we describe the influence of three NKp30 splice variants on the prognosis of gastrointestinal sarcoma (GIST), a malignancy that expresses NKp30 ligands and that is treated with NK-stimulatory KIT tyrosine kinase inhibitors. Healthy individuals and those with GIST show distinct patterns of transcription of functionally different NKp30 isoforms. In a retrospective analysis of 80 individuals with GIST, predominant expression of the immunosuppressive NKp30c isoform (over the immunostimulatory NKp30a and NKp30b isoforms) was associated with reduced survival of subjects, decreased NKp30-dependent tumor necrosis factor-α (TNF-α) and CD107a release, and defective interferon-γ (IFN-γ) and interleukin-12 (IL-12) secretion in the NK-DC cross-talk that could be restored by blocking of IL-10. Preferential NKp30c expression resulted partly from a single-nucleotide polymorphism at position 3790 in the 3′ untranslated region of the gene encoding NKp30. The genetically determined NKp30 status predicts the clinical outcomes of individuals with GIST independently from *KIT* mutation.

GIST results from the aberrant signaling from oncogenic tyrosine kinase receptors, usually the one encoded by *KIT*[1,2]. Currently, the mutational status of *KIT* is the most valuable predictive factor for therapeutic response to the KIT inhibitor imatinib mesylate[2,3]. Individuals whose tumors harbor mutations in exon 11 of *KIT* survive longer than those whose tumors express another kinase mutation or no kinase mutation. However, unexpected long-term responses to imatinib mesylate have been reported for individuals with GIST whose tumors were predicted to resist this treatment[4,5]. We have shown that the therapeutic off-target effect of imatinib mesylate is mediated by NK cells and DCs[4,6]. By inhibiting KIT in DCs, the drug can promote DC-to-NK cross-talk that ultimately stimulates NK cells to produce IFN-γ[4]. The imatinib mesylate–induced IFN-γ production by NK cells represents an independent predictor of long-term survival in advanced GIST treated with imatinib mesylate[5]. We have further analyzed GIST-associated NK cell phenotypes and detected a predominant

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downregulation of one particular type of stimulatory NK cell receptors, NKp30 (but not NKp46 or NKG2D, for instance)[5]. NKp30 expression has a key role in the prognosis of infectious diseases[7–16] and acute myeloid leukemia[17]. Indeed, NKp30, which is specifically expressed on NK cells, is involved in the killing of tumor cells and DCs[18,19].

Intrigued by the fact that NKp30 could mediate both DC killing and DC maturation[18,20], we decided to characterize NKp30 functions in individuals with GIST.

The natural cytotoxicity receptor-3 gene (*NCR3*, also known as *NKp30*, *1C7* or *LY117*), located in the highly polymorphic telomeric end of the class III major histocompatibility complex[21], is transcribed into several NKp30 mRNA splice variants, most of which are translated into cell-surface molecules of the immunoglobulin superfamily[19,22–24]. Depending on which exon 4 is translated, the immunoglobulin domain (which is either a V- or C-type domain, but forms containing the V-type immunoglobulin domain are largely overrepresented[25]) can be linked to any of three distinct intracellular domains (which have 36, 12 and 25 amino acid residues), giving rise to three isoforms: NKp30a, NKp30b and NKp30c, respectively[23]. Although *NCR1*, encoding the NKp46 receptor, has an equivalent in mice, the mouse ortholog of *NCR3* is a pseudogene (in *Mus musculus* but not in *Mus caroli*)[24–26], so there is no suitable mouse model for the exploration of NKp30.

Here we demonstrate that alternative splicing of the *NCR3* gene has a profound impact on its functions and that the distinct NKp30 isoforms can relay opposed signals that are either immunostimulatory or immunosuppressive. We show that individuals with GIST often have preferential expression of an immunosuppressive NKp30 isoform, which constitutes a biomarker that negatively affects the prognosis of GIST.

RESULTS

Natural killer cells infiltrate GIST and downregulate NKp30

Imatinib mesylate–mediated NK cell activation in individuals with GIST correlates with progression-free survival upon imatinib mesylate therapy, in the sense that patients for whom imatinib mesylate failed to induce NK cell IFN-γ secretion did not fully benefit from the therapy[5]. Moreover, at diagnosis, the major phenotypic abnormality of circulating NK cells from people with GIST is selective downregulation of NKp30 expression and function[5]. To further investigate the role of NK cells in GIST immunosurveillance, we quantified NK cells in tumor-infiltrating lymphocytes (TILs) at diagnosis using immunohistochemistry and flow cytometry. Immunohistochemical analyses of 44 GIST specimens (described in **Supplementary Table 1**) using an NKp46-specific antibody (the sole antibody suitable for *in situ* detection of NK cells) revealed substantial numbers of NK cell infiltrates, mainly localized in fibrous trabeculae surrounding tumor nests (**Fig. 1a**). The abundance of NK cells did not correlate with the Miettinen prognostic score, which is based on proliferation index, tumor size and tumor localization[27] (**Fig. 1b**). Moreover, the density of the NK cell infiltrates was not modified by imatinib mesylate therapy (**Fig. 1c**), yet it was inversely correlated with the presence of metastases at diagnosis (**Fig. 1d**). Cytofluorometric analysis of TILs at diagnosis revealed that ~25% of CD45$^+$ leukocytes were CD3$^-$CD56$^+$ NK cells (**Fig. 1e**), which is substantially more than in peripheral blood of healthy volunteers (**Fig. 1e**). Notably, all NK TILs had a CD56^{bright}CD16$^-$KIR$^-$ NK phenotype (in contrast to circulating NK cells; data not shown), and NKp30 (but not NKp46 nor NKG2D) was selectively downregulated on NK TILs (**Fig. 1f**). These results support an NK cell–mediated immunosurveillance mechanism in

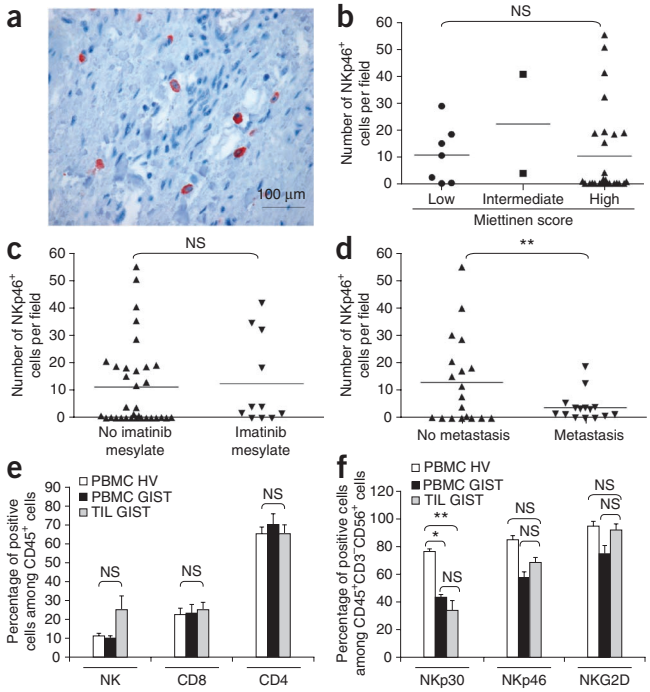

Figure 1 NKp46$^+$ TIL infiltrates in GIST inversely correlate with metastases at diagnosis. (**a**) Representative photograph from immunohistochemical analyses of NKp46$^+$ cells in 44 paraffin-embedded GIST specimens. In 70% of primary tumors, we found clusters of NKp46$^+$ cells mostly localized in fibrous trabeculae. (**b**–**d**) Mean numbers of NKp46$^+$ cells counted in five independent fields are depicted as a function of the Miettinen score (**b**), imatinib mesylate therapy (**c**) or the presence of metastases at diagnosis (**d**). (**e**,**f**) Flow cytometry analyses of CD3$^-$CD56$^+$NK cells (**f**) in CD45$^+$ live leukocytes (**e**) of freshly dissociated GIST specimens, compared with circulating blood lymphocytes of subjects with GIST or of age- and sex-matched healthy volunteers (HV). The percentages of NK cells (mean ± s.e.m., $n = 5$) expressing NKp30, NKp46 and NKG2D are shown. $^*P < 0.05$; $^{**}P < 0.01$ (Student's t test); NS, not significant.

GIST. Moreover, the selective downregulation of NKp30 *in situ* is noteworthy in view of the fact that stimulatory NK receptors can be downregulated by their ligands *in situ*[17,28]. Indeed, direct cell-cell contacts between leukemic blast cells and NK cells have been shown to induce loss or decrease in expression of natural cytotoxicity receptors in a TGF-β–independent manner[17].

Functional differences in the three NKp30 isoforms

To functionally characterize NKp30 isoforms, we cloned NKp30 splice variants that use exons 4I, 4II and 4III (referred to as NKp30c, NKp30b and NKp30a, respectively[22]) into bicistronic expression vectors together with enhanced green fluorescent protein (GFP), and we stably transfected these cDNAs into NKL cells, a human NK cell line derived from a large granular lymphocyte leukemia[29]. All transfected cells expressed comparable levels of GFP and NKp30 (**Supplementary Fig. 1a**), and, independently of the isoform, the mean fluorescence intensity of NKp30 was 20- to 30-fold higher in transfected cells than in the parental cell line. The three NKp30-transfected cell lines did not differ from parental cells in the expression of other NK cell receptors (**Supplementary Fig. 1a**).

To analyze the function of each NKp30 isoform, we first monitored the release of cytokines after NKp30 engagement. NKL cells expressing either NKp30a or NKp30b produced large amounts of IFN-γ, whereas

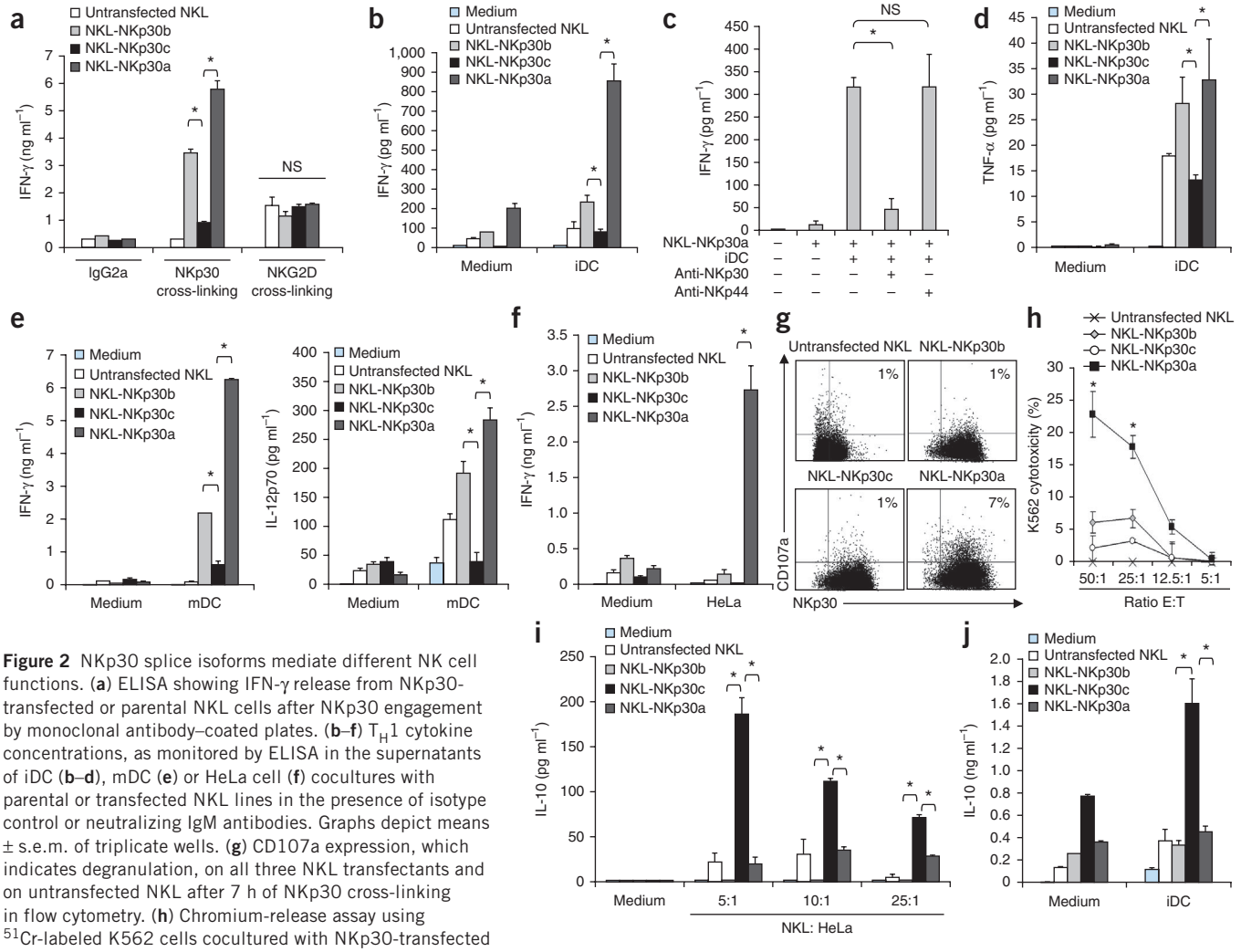

Figure 2 NKp30 splice isoforms mediate different NK cell functions. (**a**) ELISA showing IFN-γ release from NKp30-transfected or parental NKL cells after NKp30 engagement by monoclonal antibody–coated plates. (**b**–**f**) T_H1 cytokine concentrations, as monitored by ELISA in the supernatants of iDC (**b**–**d**), mDC (**e**) or HeLa cell (**f**) cocultures with parental or transfected NKL lines in the presence of isotype control or neutralizing IgM antibodies. Graphs depict means ± s.e.m. of triplicate wells. (**g**) CD107a expression, which indicates degranulation, on all three NKL transfectants and on untransfected NKL after 7 h of NKp30 cross-linking in flow cytometry. (**h**) Chromium-release assay using ^{51}Cr-labeled K562 cells cocultured with NKp30-transfected and untransfected NKL cells at various effector to target (E:T) ratios. (**i**,**j**) ELISAs showing IL-10 production after engagement of the NKp30c isoform as in **b**,**f**. Experiments in **b**–**e**,**j** were performed five times using three different DC donors, yielding similar results. $^*P < 0.05$; NS, not significant. Error bars represent means ± s.e.m. of triplicate wells.

cells expressing the NKp30c isoform did not (**Fig. 2a**). Similar results pointing to a stimulatory function of NKp30a and NKp30b, but not NKp30c, were obtained when Jurkat cells transfected with NKp30 isoforms were examined for IL-2 production (data not shown). All transfected NKL cell lines produced equal amounts of IFN-γ when stimulated with high doses of IL-2, phorbol-12-myristate-13-acetate and ionomycin or with NKG2D-specific antibodies, excluding a nonspecific perturbation of cellular signaling by the NKp30 isoforms (**Fig. 2a** and **Supplementary Fig. 1b**). NKL cells transfected with NKp30c (NKL-NKp30c) did not recognize immature DCs (iDCs), in contrast to NKL-NKp30a or NKL-NKp30b cells, which readily secreted IFN-γ and TNF-α upon coculture with iDCs in an NKp30- but not NKp44-dependent manner (**Fig. 2b–d**). Intracellular staining revealed that both T helper type 1 (T_H1) cytokines (IFN-γ and TNF-α) were produced in NKL-NKp30a cells by 18 h and in NKL-NKp30b cells by 24 h upon coculture with iDCs (**Supplementary Fig. 2**). Moreover, the cross-talk between mature DCs (mDCs) and transfected NKL cells led to high IFN-γ secretion by NKL cells and IL-12p70 production by mDCs cocultured with NKL-NKp30a cells; lower amounts of such T_H1 cytokines were produced with NKL-NKp30b cells, and very low or undetectable levels were produced with NKL-NKp30c cells (**Fig. 2e**).

Cocultures of several tumor cell lines (which express the NKp30 ligand B7-H6; ref. 30) with all three transfected NKL cell lines and the parental NKL line revealed that NKL-NKp30a and, to a lesser extent, NKL-NKp30b responded to tumor cells by producing IFN-γ (**Fig. 2f** and **Supplementary Fig. 1c**). NKL-NKp30a was the only line able to block tumor cell proliferation (**Supplementary Fig. 3a**), and this line also showed NK granule exocytosis leading to the expression of CD107a at the cell surface (**Fig. 2g**) and mediated cytotoxic effects against K562 or P815 cells (**Fig. 2h** and **Supplementary Fig. 3b**).

Ligation of NKp30 can trigger the canonical pathway of nuclear factor-κB (NF-κB) activation, which depends on the phosphorylation and subsequent degradation of inhibitor of κBα (IκBα)[31]. Phosphorylation and degradation of IκBα occurred rapidly within 2 h in NKL-NKp30a cells stimulated by NKp30 cross-linking (**Supplementary Fig. 4a**,**b**), leading to the translocation of NF-κB p65 into the nucleus within 60 min, as well as to its DNA binding (**Supplementary Fig. 4c**,**d**). The degradation of IκBα occurred with slower kinetics for NKp30-stimulated NKL-NKp30b and seemed incomplete with NKL-NKp30c even at late time points (240 min) (**Supplementary Fig. 4a**,**b**). In contrast, all NKp30 transfectants similarly activated NF-κB in response to TNF-α (**Supplementary Fig. 4a**,**c**,**d**).

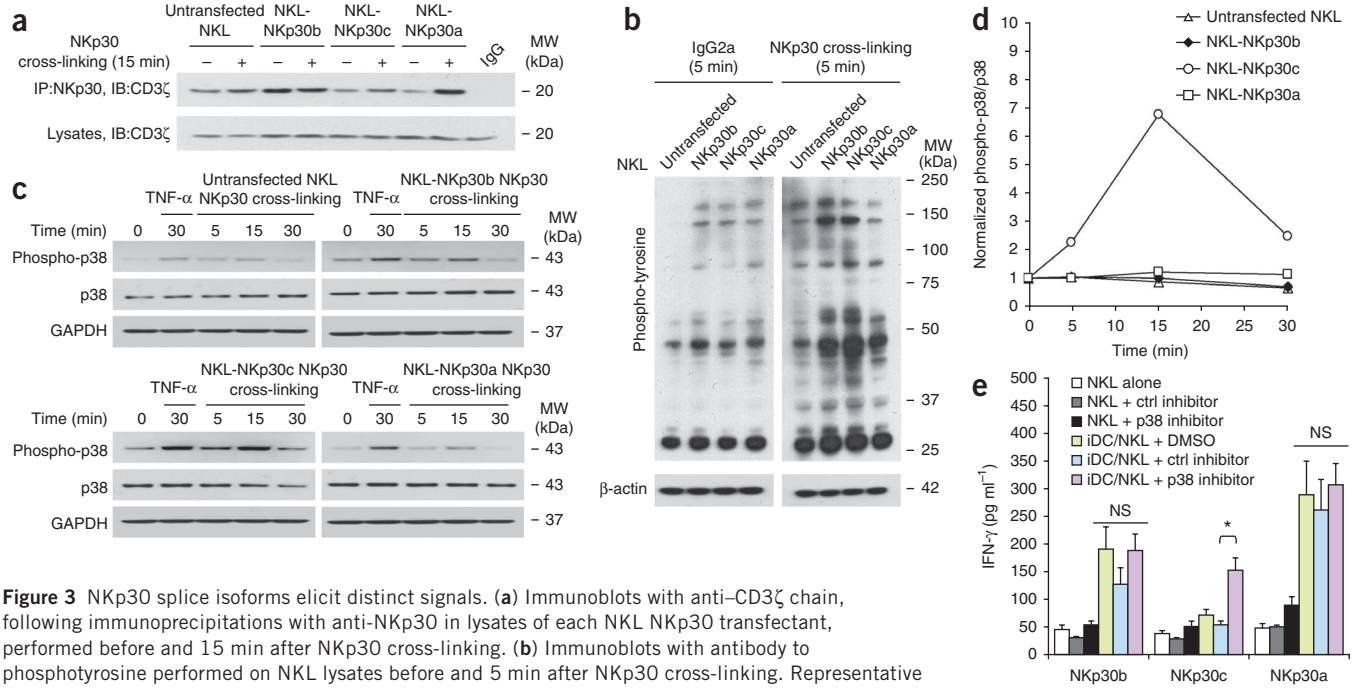

Figure 3 NKp30 splice isoforms elicit distinct signals. (**a**) Immunoblots with anti–CD3ζ chain, following immunoprecipitations with anti-NKp30 in lysates of each NKL NKp30 transfectant, performed before and 15 min after NKp30 cross-linking. (**b**) Immunoblots with antibody to phosphotyrosine performed on NKL lysates before and 5 min after NKp30 cross-linking. Representative blots are shown; similar results were obtained for three distinct clones of each NKp30 transfectant in six experiments. (**c**) Immunoblots showing p38 MAPK activation in the three NKL-transfected cell lines at different time points after NKp30 cross-linking. Immunoblotting was performed on NKL cell lysates using specific antibodies for phosphorylated p38 (Phospho-p38), total p38 (p38) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One representative immunoblot of three is shown. (**d**) Phospho-p38/p38 ratios from **c** normalized to GAPDH. (**e**) IFN-γ levels (mean ± s.e.m.) in NKp30-transfected NKL cells preincubated with a pharmacological inhibitor of p38 MAPK (SB203580) or a chemically related mock inhibitor (SB202474), then cocultured with iDC at a 1:1 ratio. The experiments were performed three times, yielding similar results. $^*P < 0.05$; NS, not significant. Error bars represent means ± s.e.m. of triplicate wells.

In contrast to NKL-NKp30a or NKL-NKp30b cells, NKL-NKp30c cells could produce significant (but relatively low) levels of the inhibitory cytokine IL-10 upon coculture with B7-H6–expressing tumor cells (**Fig. 2i** and **Supplementary Fig. 3c**) or iDCs (**Fig. 2j** and **Supplementary Fig. 2**). This was corroborated by monitoring of the amount of IL-10–specific mRNA produced by NKL-NKp30c cells (data not shown). NKp30 transduces its downstream signal through adapters including CD3ζ (ref. 19). Upon cross-linking, the NKp30a isoform associated with CD3ζ, whereas the NKp30c isoform showed a less tight association. In contrast, the NKp30b isoform constitutively associated with CD3ζ (**Fig. 3a**). All three isoforms were able to mediate increased protein phosphorylation on tyrosines, although with quantitative differences (**Fig. 3b**). Next, we explored the NKp30-driven activation of mitogen-activated protein (MAP) kinases[32]. The activation of p38 MAP kinase, which peaked at 15 min, was more pronounced in NKL-NKp30c cells than in NKL-NKp30a or NKL-NKp30b cells (**Fig. 3c**,**d**), whereas no differences were observed for extracellular signal–regulated-1/2 MAP kinases (data not shown). Notably, p38 MAP kinase inhibitors restored IFN-γ production by NKL-NKp30c cells stimulated with iDCs (**Fig. 3e**) or tumor cells (**Supplementary Fig. 4e**).

In conclusion, the three NKp30 isoforms transmit distinct signals. NKp30a is the only isoform that triggers cytotoxicity, whereas both NKp30a and NKp30b stimulate T_H1 cytokine release (IL-12 from DCs and IFN-γ from NK cells). In contrast to these two immunostimulatory NKp30 isoforms, NKp30c promotes IL-10 production, relaying an immunosuppressive signal through a rapid phosphorylation of p38 MAP kinase.

Expression patterns of NKp30 isoforms in GIST

To investigate the expression of NKp30 isoforms in peripheral NK cells, we performed quantitative RT-PCR (qRT-PCR) using primers specific for NKp30a, NKp30b or NKp30c. We normalized the expression levels to β2 microglobulin on bulk peripheral blood mononuclear cells (PBMCs) from subjects with GIST or healthy individuals. Unsupervised hierarchical clustering of expression data from 80 subjects with GIST classified 44 as 'profile AB', with NKp30a or NKp30b as the most abundant isoform, and 36 as 'profile C', with NKp30c the most abundant (**Fig. 4a**,**b**). The results of NKp30-specific qRT-PCR were comparable for PBMCs and purified NK cells (**Supplementary Fig. 5a**). Moreover, the relative expression of NKp30 isoforms was stable over time, irrespective of the disease and its treatment (**Supplementary Fig. 5b**). The NKp30 expression profile was also similar in various human organs (lymph nodes, liver and blood; **Supplementary Fig. 5c**).

We compared the NKp30 expression profiles from subjects affected by GIST with those from 56 healthy volunteers who were matched in sex, age and geographic origin. PBMCs or NK cells from healthy volunteers predominantly expressed the NKp30a and NKp30b isoforms, thus resembling profile-AB subjects with GIST (**Fig. 4b**). Classification of the NKp30 expression profiles from 56 healthy volunteers using the clustering established for subjects with GIST showed that the affected subjects and the healthy volunteers differed in the proportion of individuals with predominant NKp30c expression. Whereas 53% of subjects with GIST had profile C, only 30% of healthy volunteers fell into this category (Fisher's exact test, $P = 0.02$).

Together, these results indicate that stable phenotypes arise from the preferential exon 4 splicing of NKp30 transcripts, and that preferential

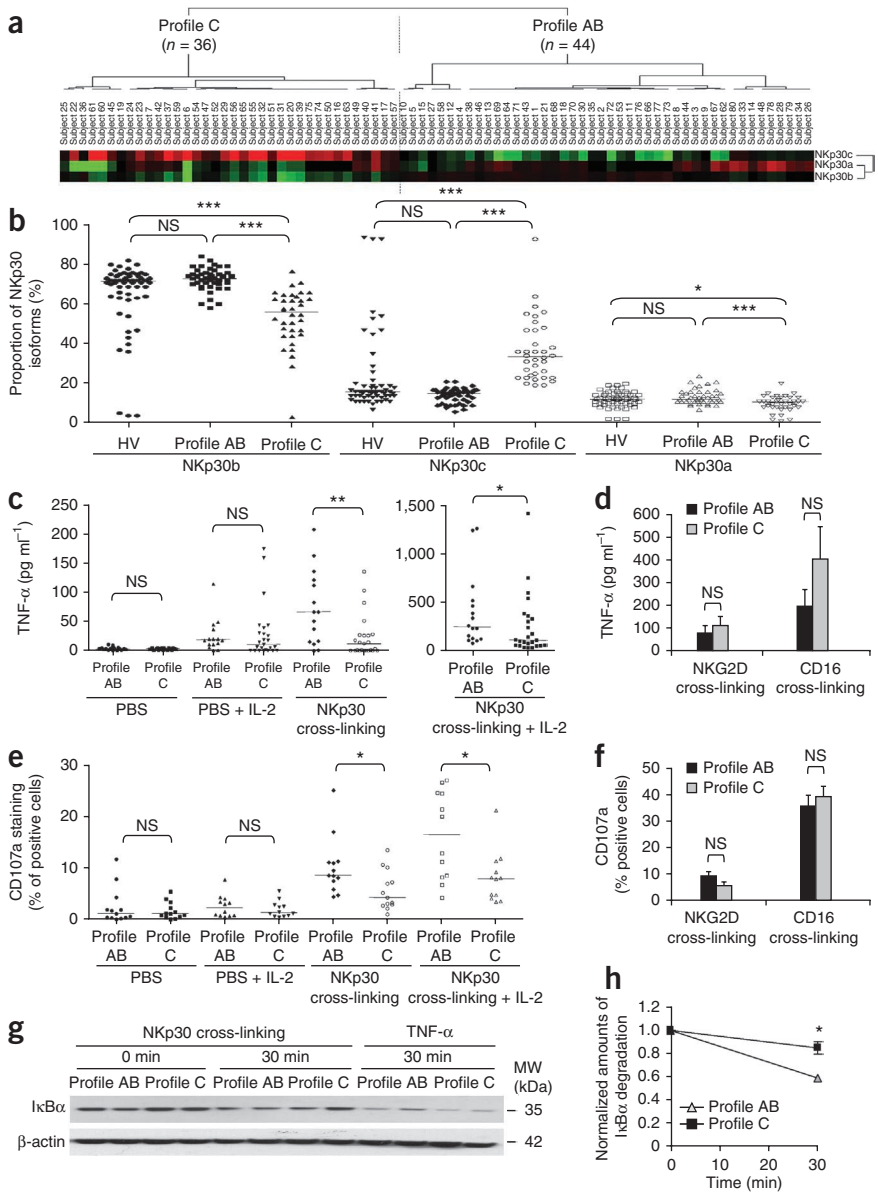

Figure 4 Expression of NKp30 isoforms in a cohort of individuals with GIST. (**a**) NKp30 expression profiles from a cohort of individuals with GIST ($n = 80$). The relative expression of the three NKp30 isoforms was determined by qRT-PCR, and unsupervised hierarchical clustering of NKp30 profiles was applied. Each row represents one NKp30 isoform and each column represents one subject. Red and green colors indicate expression levels above and below the median, respectively. (**b**) Proportions of NKp30a, NKp30b and NKp30c isoforms obtained from the hierarchical clustering, for profile-AB ($n = 44$) and profile-C ($n = 36$) individuals with GIST and for a cohort of healthy volunteers (HV; $n = 56$). Medians are shown. (**c**–**f**) ELISAs showing TNF-α secretion (**c**,**d**) and flow cytometry analyses of CD107a expression (**e**,**f**) after cross-linking of NKp30 ($n = 40$), CD16 or NKG2D ($n = 12$) in the presence or absence of IL-2 on circulating NK cells from individuals with GIST. Data are means ± s.e.m. of triplicate wells for each individual. (**g**) IκBα degradation on purified NK cells from profile-AB and profile-C individuals stimulated with TNF-α or NKp30 cross-linking for 30 min. One representative immunoblot is shown from two individuals out of four tested. (**h**) IκBα degradation in **g** normalized to β-actin levels ($t = 0$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. Data represent mean ratios of two independent experiments.

expression of the immunosuppressive NKp30c isoform (profile C) is more frequent in individuals with GIST than in the control group.

Natural killer cells are defective in profile-C GIST

NK cells purified from individuals with GIST who had profile AB or C were subjected to NKp30 cross-linking, and TNF-α release and degranulation were measured. NK cells from profile-C individuals showed a significant decrease in TNF-α production and CD107a expression, as compared to profile-AB NK cells (**Fig. 4c**–**e**), in accordance with the results obtained in NKL cells. These functional defects observed for profile-C individuals were NKp30 specific, as there were no significant differences in TNF-α and CD107a expression between profile-AB and profile-C NK cells upon stimulation with other agents (**Fig. 4d**,**f**). NK cells from profile-C individuals also showed a delay in IκBα degradation, compared with profile-AB individuals, after NKp30 cross-linking (but not after TNF-α stimulation; **Fig. 4g**,**h**).

Next, we analyzed the differential capacity of NK cells purified from profile-AB and profile-C individuals to respond to autologous mDCs. T_H1 cytokine release was significantly compromised in profile-C individuals (**Fig. 5a**). IL-10 release from NKp30c-expressing NK cells (upon encounter with the NKp30-specific ligand on DCs) might prevent IL-12 secretion by autologous DCs, therefore abrogating IFN-γ release by NK cells, in line with the previously reported immunosuppressive function of IL-10–producing NK cells[33,34]. Indeed, blocking IL-10 (or its receptor) markedly enhanced the production of both IFN-γ and IL12p70 during the NK-to-mDC cross-talk—particularly in profile-C individuals (**Fig. 5b**)—in an NKp30- but not NKp44-dependent manner (**Fig. 5c**). Hence, profile-C individuals have an overt yet selective defect in NKp30-driven NK effector functions.

Poor prognosis of profile-C GIST

We performed a retrospective analysis of the influence of NKp30 isoforms on the overall survival of 80 imatinib mesylate–treated individuals with GIST, with a mean follow-up of 58 months (12 to 103 months). Subject groups with profiles AB and C did not differ significantly ($P > 0.05$) in any of the classical parameters dictating GIST prognosis, including KIT mutations (**Supplementary Table 2**)[2,35]. Nonetheless, profile-C individuals had reduced overall survival from initial imatinib mesylate treatment in univariate analysis (median survival of 79 months in profile C, compared with median not reached in profile AB; log-rank test, $P = 0.001$; **Fig. 6a**). In multivariate analyses using the Cox model, profile C was retained as the sole independent prognostic factor for overall survival (relative risk = 13.1, 95% confidence interval 4.9–36.1, $P = 0.01$). The analysis of overall survival after discontinuation of imatinib mesylate revealed 15 deaths resulting from 21 relapses at 50 months in profile-C

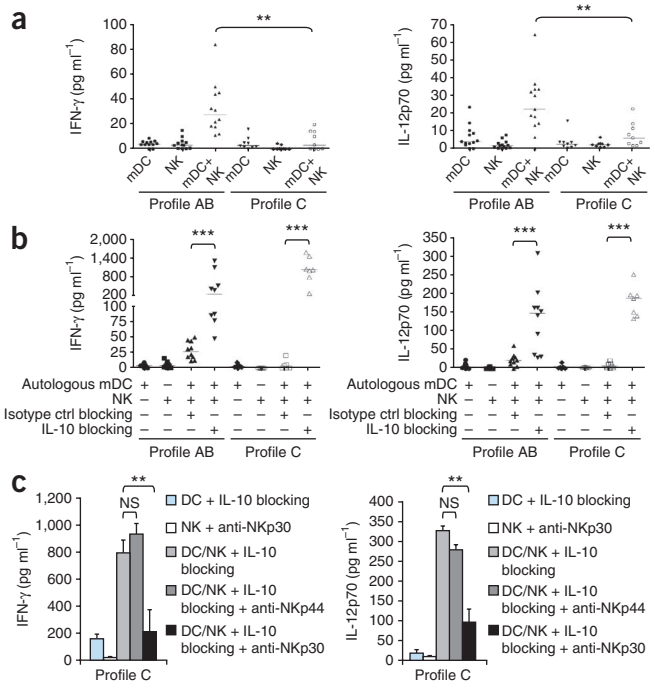

Figure 5 Neutralization of IL-10 restores T_H1 cytokine production during the interaction of DC and NK cells from profile-C individuals with GIST. (**a**) IFN-γ and IL-12p70 secretion detected in autologous mDC-NK cross-talk from profile-AB ($n = 12$) and profile-C ($n = 9$) individuals with GIST. (**b**,**c**) IFN-γ and IL-12p70 secretion in autologous mDC-NK cross-talk from profile-AB ($n = 10$) or profile-C ($n = 7$) individuals, with cells cocultured as in **a** but in the presence of neutralizing antibody to IL-10 and IgM antibody to NKp30 or NKp44. Similar results were obtained using anti–IL-10R. Results from one representative subject (profile C) out of two are depicted in **c**; means ± s.e.m. are shown in **c**. $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant.

individuals, compared to only five deaths out of 20 relapses in profile AB individuals (log-rank test, $P = 0.006$; **Fig. 6b**). Although most subjects with GIST showed decreased membrane expression of NKp30 compared with healthy volunteers, independently of NKp30 profile (**Fig. 6c**), this parameter had no impact on overall survival (**Fig. 6d**,**e**). Moreover, the NKp30 profile did not affect the density of intratumoral NK infiltration (**Fig. 6f**). In summary, preferential expression of the immunosuppressive NKp30c isoform (profile C) predicts an unfavorable therapeutic outcome, presumably because it negatively affects NK cell function without affecting the expression of NK cell markers or the frequency of NK cells.

NCR3*3790 and NCR3*3918 mutations and profile C

We subsequently correlated the preferential expression of NKp30 splice variants with single-nucleotide polymorphisms (SNPs) affecting this gene. Out of 21 SNPs recorded in the databases for the *NCR3* gene, we preferentially studied those residing in the promoter, exons or 5 untranslated region occurring at a frequency of >5%. A SNP (NCR3*3790 T/C, rs986475) located in polyadenylation signal 2 (PS2) of the *NCR3* gene compromised the transcription of NKp30a and NKp30b owing to a single-nucleotide substitution within the AAUAAA motif, which is predicted to affect polyadenylation and cleavage efficiencies[36]. No profile-AB individuals had the NCR3*3790 TC or CC genotypes, but 51% of profile-C individuals and 58% of healthy volunteers had these genotypes (Fisher's exact test, $P < 0.0001$; **Supplementary Table 3**). As NCR3*3790 T/C accounts for the overexpression of NKp30c in only half of profile-C individuals,

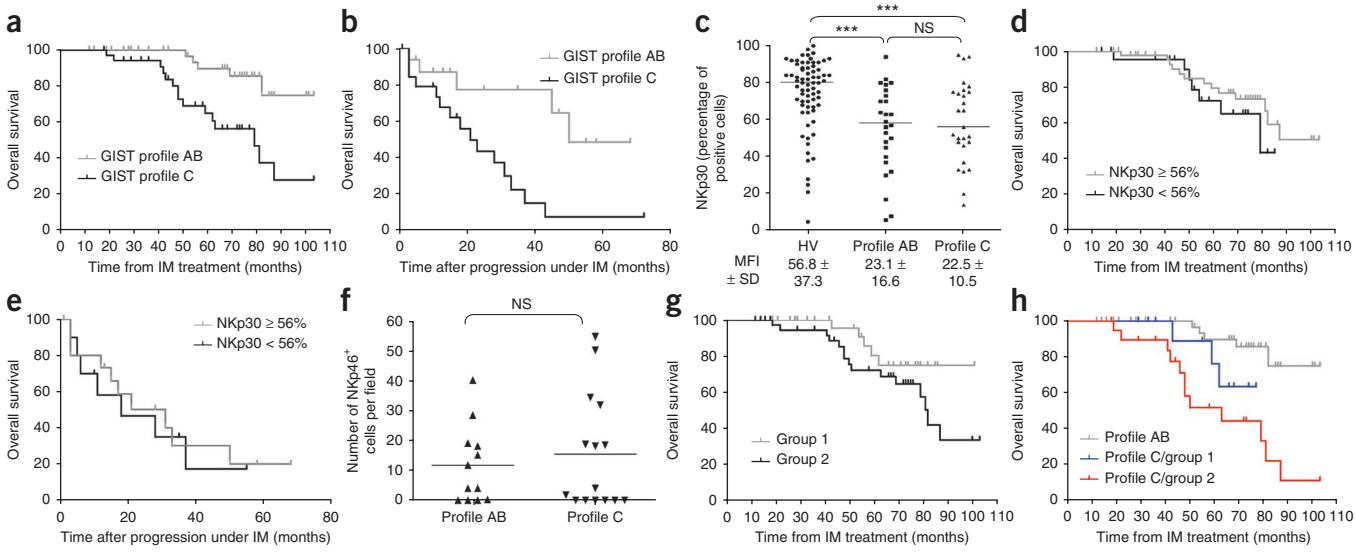

Figure 6 NKp30 isoform profiles predict the survival of subjects with GIST. (**a**) Overall survival from initial imatinib mesylate treatment in profile-AB ($n = 44$) compared with profile-C ($n = 36$) individuals with GIST, assessed in univariate analysis using the Kaplan-Meier method. The log-rank test was used. (**b**) Overall survival after progression in profile-AB ($n = 20$) and profile-C ($n = 21$) individuals with GIST. (**c**) Flow cytometry results showing NKp30 expression in subjects with profiles AB and C (shown in percentages and mean fluorescence intensity (MFI)). Fresh NK cells were examined at the time of the transcriptional analysis of the NKp30 isoforms during imatinib mesylate therapy. (**d**,**e**) Analyses as in **a**,**b** comparing overall survival of subjects with GIST who differed in NKp30 surface expression (measured by flow cytometry). The subjects' NKp30 expression was <56% ($n = 24$) or ≥56% ($n = 29$) on circulating NK cells at the time of transcriptional profiling; the 56% threshold was the median of NKp30 expression for the 53 subjects in **c**. (**f**) The abundance of $NKp46^+$ cell infiltrates in GIST, plotted against subjects' transcriptional profiles. The 27 GIST specimens are described in **Supplementary Table 1**. (**g**) Overall survival for NCR3*3790/3918 haplotypic group 1 (NCR3*3790/3918 TA and CT, $n = 32$) and group 2 (NCR3*3790/3918 CA and TT, $n = 22$). (**h**) Overall survival based on the combination of the NCR3*3790/3918 haplotypic groups and NKp30 transcriptional profile. $^{***}P < 0.001$; NS, not significant.

we performed a logistic regression analysis on the 11 selected SNPs. The transcriptional NKp30 phenotype could be modeled by three SNPs: NCR3*−204 T/C, NCR3*3790 T/C and NCR3*3918 A/T. As the NCR3*−204 T/C SNP is in linkage disequilibrium with NCR3*3918 A/T, the most restrictive genetic model capable of explaining the transcriptional pattern was based on NCR3*3790 T/C and NCR3*3918 A/T SNPs, representing two complementary haplotypes. Taken alone, these haplotypic groups were not as potent as the transcriptional profiles in predicting overall survival (**Fig. 6g**). However, the stratification of profile-C individuals into the two haplotytic groups led to the identification of a subgroup of profile C (haplotypic group 2) with dismal prognosis (**Fig. 6h**).

DISCUSSION

Here we have characterized NKp30 splice isoforms and revealed the predictive value of genetic and transcriptional profiles of NKp30, thus identifying a subgroup of individuals with GIST who have an unfavorable prognosis. Our results suggest NKp30 isoforms have contrasting effects and can be functionally classified as two immunostimulatory isoforms (NKp30a, NKp30b) and one immunosuppressive isoform (NKp30c). Although all three isoforms associated with CD3ζ, only NKp30c could induce the activating phosphorylation of p38 MAPK, and pharmacological inhibition of p38 MAPK partially restored the immunostimulatory activity of NKp30c. We also addressed whether each NK cell expresses all isoforms or whether the isoforms are expressed on defined NK subpopulations. NK clones from distinct individuals each expressed all three isoforms (**Supplementary Fig. 5d**). Thus, it seems plausible that in profile-C individuals, most NK cells relay an inhibitory rather than a stimulatory signal upon interaction with NKp30 ligands.

NKp30 isoform expression patterns were not influenced *in vitro* by NK-stimulatory or NK-inhibitory cytokines or by imatinib mesylate; instead, our data indicate that these expression patterns are strongly controlled by genetic factors. We have identified one such genetic factor, a constellation of SNPs (including the NCR3*3790 SNP) that explains the relative overexpression of the immunosuppressive NKp30c isoform among half of the profile-C individuals.

Extending our previous data[4,5], this study offers further evidence of the relevance of NK cells in the control of GIST. The density of NK cell infiltration inversely correlated with the presence of metastases at diagnosis. qRT-PCR and flow cytometry analyses reveal that more than 25% of GIST specimens express B7-H6, a ligand for NKp30 (refs. 30,37 and data not shown). It is therefore tempting to speculate that the DC-NK dialog might not lead to T_H1 cytokine secretion in profile-C individuals, reducing the priming of naïve NK cells and/or T cell responses[38], and IL-10 production by NKp30c-expressing NK cells might instead promote tumor cell survival and/or metastases.

Individuals with GIST who preferentially expressed NKp30c on their NK cells (profile C) normally responded to targeted therapy with imatinib mesylate for the first 3 years, but many of them died later on, presumably because the drug had lost its antiproliferative effect, owing to additional KIT mutations, at the same time that it has no immunological off-target effect. Blocking IL-10 (or IL-10R) with specific antibodies may improve the long-term efficacy of imatinib mesylate in profile-C individuals. Alternatively, enhancing NK cell function with immunostimulatory cytokines (such as IL-2, IL-15, IL-21, Flt3 ligand or type 1 interferon) or by neutralization of the immunosuppressive IL-23/IL-23 receptor system[39] might further ameliorate the outcome of GIST in profile-AB individuals. Subclassifying metastatic GIST on the basis of the NKp30 profile may help in designing innovative clinical trials. Thus, beyond its utility as a biomarker, the mutational status of the NKp30 locus and its expression profile may guide a new type of NK-centered, GIST-specific immunotherapy.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

N.F.D., S. Ruskiewicz, I.M., C.M., S. Roux, N.C., M. Sarabi, C.F., M. Semeraro, V.P.-C. and K.C. performed the experiments. L.L. and P.P. did qRT-PCRs. V.M.-C. and D.V.-C. contributed to clinical aspects of the study. V.B. and H.A. performed the electrophoretic mobility shift assays. S.K.-R. and M.P. provided the p38 MAP kinase inhibitors and scientific advice. I.C. contributed to the NKp46 immunohistochemistry study. L.P. and B.R. performed single-cell RT-PCRs. C.B. and A.M. provided the NK cell clones and the NKp30 and NKp44 antibodies. J.C.G., J.A.N. and J.T. contributed to exploration of the MAP kinase pathway. F.C. contributed to the statistical analysis. N. Ibrahim, P.T. and P.O. provided the paraffin-embedded GIST specimens and monitored histopathology data. S.B., J.-M.C., J.-Y.B., N. Isambert and A.L. provided samples from affected individuals and clinical data. J.-F.E. identified the KIT mutations in the affected-subject cohort. P.R. contributed to genotyping of the *NCR3* mutations. E.V. provided the B7H6 antibody and scientific advice. N.F.D., S. Rusakiewicz and I.M. prepared the figures and drafted the manuscript. G.K. and L.Z. designed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects with GIST and healthy volunteers. The characteristics of the 44 paraffin-embedded GIST specimens subjected to immunohistochemistal analysis are described in **Supplementary Table 1**. The immunomonitoring studies were performed prospectively in people with GIST enrolled in the EORTC phase 3 trial 62005 and in the French Sarcoma Group phase 3 clinical trial (BFR14), assessing dose and duration of imatinibmesylate, respectively. Informed written consent was obtained from subjects according to guidelines of local ethical committees. Heparinized blood was drawn from subjects during treatment (between 2 to 94 months of imatinibmesylate therapy, mean 28 ± 3 months). Subjects' characteristics are summarized in **Supplementary Table 2**. Clinical responses were assessed by computed tomography scan and the responses were classified according to the RECIST criteria. Fifty-six healthy volunteers (sex- and age-matched with subjects affected by GIST) were used as controls for the immunological parameters.

DC-NK cross-talk *in vitro*. T_H1 cytokine concentrations (mean ± s.e.m.) after iDCs, mDCs (obtained from iDCs cultured with CD40 ligand and lipopolysaccharide) or HeLa tumor cells were mixed with untransfected NKL cells (3:1 NKL cells:DCs or tumor cells), with NKL cells overexpressing all three NKp30 isoforms (1:1 NKL cells:DCs or tumor cells) or with medium alone, for 24 h in the presence of isotype control or neutralizing IgM antibodies (anti-NKp44 or anti-NKp30).

Quantitative reverse-transcription PCR and nonhierarchical clustering. Total cellular RNA was isolated from PBMCs, purified NK, NK clones[40] and NKL cell lines with the RNeasy kit. First-strand cDNA was synthesized from 5 µg of total RNA using SuperScript III reverse transcriptase and random primers according to Invitrogen's instructions. The PCR primers and TaqMan probes for the six NKp30 transcripts and the β2 microglobulin housekeeping transcript were designed with Primer Express software version 1.0 (Applied Biosystems) and are listed in the **Supplementary Methods**. For each NKp30 isoform, standard curves based on six data points of NK92 cDNA dilutions in triplicates were established to estimate the efficiency of PCR amplifications with the StepOne software version 2.0. The efficiencies of NKp30a, NKp30b and NKp30c PCR were 88.8%, 90.6% and 93.8%, respectively. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method[41], according to the manufacturer's recommendations. The proportions of the distinct NKp30 isoforms were determined as the ratio of the relative quantities of each isoform and the total quantity of the three isoforms. Unsupervised hierarchical clustering was applied to data that had been log-transformed and median-centered using the Cluster and TreeView programs (average linkage clustering using Pearson's centered correlation as similarity metric)[42].

Genotyping of *NCR3*. We sequenced the three PCR products as previously described[43]. Briefly, the three fragments were amplified by PCR (primer sequences are listed in **Supplementary Methods**), PCR products were purified with the QiagenQIAquick PCR purification kit and quantified by 2% agarose gel electrophoresis, and sequencing reactions were performed with the Cequation (8000) kit and a Cequation (8000) automated fluorescence sequencer.

The NCR3*3790 T/C (rs986475) and NCR3*3918 A/T (rs1052248) genotypes were confirmed with the TaqMan genotyping assays C_7514908_10 and C_2451901_10, respectively. Briefly, 10 ng of genomic DNA was mixed with 5 µl of 2×TaqMan Genotyping Master Mix and 0.25 µl of 40× genotyping assay buffer in a final volume of 10 µl. Temperature cycling and real-time fluorescence measurement were done using the StepOnePlus System.

Immunoblotting and CD3ζ immunoprecipitation. Phosphorylation of IκBα, tyrosine residues and p38 MAP kinase was detected by immunoblot analysis. Briefly, 2×10^6 resting (IL-2 starved) NKL-NKp30 transfectants or 1×10^6 purified NK cells were seeded (2×10^5 NK per well) in 96-well Maxisorp plates coated with 2.5 µg ml^{-1} of mouse IgG2a NKp30-specific antibody (clone 210847) or isotype control. The cells were incubated with TNF-α (10 ng ml^{-1}) or sodium orthopervanadate (100 µM) as a positive control. NK cells were washed in cold PBS and lysed in sample buffer containing 1% vol/vol NP40, 20 mM HEPES (pH 7.9), 10 mMKCl, 1 mM EDTA, 1 mM PMSF, 1% vol/vol glycerol, and protease and phosphatase inhibitors. We separated 15 µg proteins on 10% SDS-PAGE gels and electrotransferred them to Immobilon membranes. After blocking with 3% wt/vol BSA and 0.1% vol/vol Tween 20, we used primary antibodiesspecific for the following proteins: phospho-IκB (clone 5A5, mouse antibody 9246), IκBα (antibody 9242), phospho-tyrosine phospho-p38 (Thr180/Tyr182) (clone 3D7, rabbit monoclonal antibody 9215), phosphotyrosine (16-316, 4G10R Platinum)), p38a MAP kinase (clone 7D6, rabbit monoclonal antibody 2371), horseradish peroxidase conjugate NKp30 (sc-20477NKp30, clone G-19). Primary antibodies that specifically recognize GAPDH (MAB374, clone 6C5) or β-actin (MAB1501, clone C4) were used as loading controls. Finally, bound antibodies were detected with the appropriate horseradish peroxidase–labeled secondary antibodies and ECL Plus detection system. For the CD3ζ immunoprecipitation, 90×10^6 resting (IL-2 starved) NKp30-transfected or untransfected NKL, before or after 15 min NKp30 cross-linking, were collected and lysed in 1% wt/vol digitonin buffer, precleared with protein G–Sepharose and coupled with NKp30-specific antibody (clone G-19) overnight at 4 °C. Immunoprecipitates were dephosphorylated with 10 U of calf intestine alkaline phosphatase in 40 µl CIP reaction buffer for 1 h, separated on 10% SDS-PAGE gels and probed with CD3ζ-specific antibody (clone 2H2), followed by rabbit anti–mouse–horseradish peroxidase. The purified goat IgG was used as a control.

Statistical analyses. The Fisher's exact test, the χ^2 test and the nonparametric Mann-Whitney test were used for comparison of the different groups. These statistical analyses were performed with the GraphPad Prism software version 5. The survival curves were plotted according to the Kaplan-Meier method and compared using the log-rank test. Independent risk factors for overall and progression-free survivals were determined using the Cox proportional hazards regression model. For survival analysis, the SPSS 16.0 software was used.

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RESEARCH PAPER

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Analysis of NKp30/NCR3 isoforms in untreated HIV-1-infected patients from the ANRS SEROCO cohort

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Keywords: HIV-1, immunosurveillance, natural course of infection, NK, NKp30 isoforms

Abbreviations: DC, dendritic cell; GIST, gastrointestinal stromal tumor; HD, healthy donor; KIR, killer-cell immunoglobulin-like receptor; MFI, mean fluorescence intensity; NCR, natural cytotoxicity receptors; NK, natural killer; PBMC, peripheral blood mononuclear cell

Natural killer (NK) cells play a prominent role at the intersection between innate and cognate immunity, thus influencing the development of multiple pathological conditions including HIV-1-induced AIDS. Not only NK cells directly kill HIV-1-infected cells, but also control the maturation and/or elimination of dendritic cells (DCs). These functions are regulated by the delicate balance between activating and inhibiting receptors expressed at the NK-cell surface. Among the former, NKp30 has raised significant interest since the alternative splicing of its intracellular domain leads to differential effector functions, dictating the prognosis of patients bearing gastrointestinal sarcoma, and B7-H6 has recently been identified as its main ligand. Since NKp30 is downregulated in $CD56^{-}/CD16^{+}$ NK cells expanded in viremic, chronically infected HIV-1^{+} patients, we decided to investigate the predictive value of NKp30 splice variants for spontaneous disease progression in 89 therapy-naïve HIV-1-infected individuals enrolled in an historical cohort of patients followed since diagnosis (ANRS SEROCO cohort). We found no difference in the representation of NK-cell subsets ($CD56^{bright}$, $CD56^{dim}$, $CD56^{neg}$) in HIV-1-infected patients as compared with healthy subjects. NKp30 downregulation was detected in $CD56^{dim}$ and $CD56^{neg}$ NK-cell subsets, yet this did not convey any prognostic value. None of the NKp30 isoforms did affect disease progression, as measured in terms of time-to-loss of circulating $CD4^{+}$ T cells, time-to-AIDS-defining events and overall survival. NKp30 isoforms do not seem to play a major role in the outcome of HIV-1 infection, but the heterogeneity of the immuno-virological status of patients at enrollment could have to be taken into account.

Introduction

Accumulating evidence suggests an important role for innate immunity in the control of acute HIV-1 infection prior to the establishment of adaptive immune responses, as well as in the subsequent rate of viral replication and disease progression.[1]

A vast array of receptors with either inhibitory or activating functions regulates the interaction between natural killer (NK) cells and other cells. Uninfected and untransformed "self" cells are recognized by inhibitory NK-cell receptors that sense normal HLA Class I molecules expression levels and prevent NK-cell activation.[2] Killer-cell immunoglobulin-like receptors (KIRs) are the main receptors for HLA Class I molecules (i.e., HLA-A, HLA-B, HLA-C and HLA-E).[2] The major NK-cell activating molecules include natural cytotoxicity receptors (NCRs) (i.e., NKp46, NKp30 and NKp44) and NKG2D, which are readily triggered by ligands expressed at the surface of infected and transformed cells.[3] The activating NK-cell receptor NKp30 is involved in both dendritic cells (DC) killing and DC maturation,[4] and appears not only to be critical for tumor-cell recognition[5] but also to influence the prognosis of different infectious diseases.[6–12] The human NKp30-encoding gene (*NCR3*) is transcribed in six different splice variants,[13] among which the most highly expressed are NKp30a, b and c.[14]

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The significance of NK-cell antiviral activity in vivo is indicated by the fact that HIV-1 evolved specific strategies to evade NK-cell responses. Indeed, the viral protein Nef acts on infected cells by selectively downregulating the expression of HLA-A and HLA-B (preventing cells to be recognized and eliminated by T cells), but not of HLA-C and HLA-E (protecting cells from NK-cell cytotoxicity).[15] Nef also induces the downregulation of ligands for the activating NK receptors NKG2D (i.e., MICA, ULBP1 and ULBP2)[16] and NKp44.[17] During chronic infection, HIV-1 mutants are detected that enhance the binding of the inhibitory receptor KIR2DL2 to its ligands, thus avoiding the recognition of infected cells by NK cells.[18] The relative amounts of activating and inhibitory KIRs play a role in the containment of viral replication in HIV-1-infected individuals.[19] Furthermore, NK cells seem to be relevant determinants for the outcome of HIV-1 infection, as the deletion of the gene encoding the NK-cell activating receptor NKG2C is a risk factor for HIV-1 infection,[20] and increased NK-cell activity has been correlated with protection from infection in several cohorts of highly-exposed seronegative subjects.[21]

HIV-1 infection is associated with a functional impairment of NK cells that is evident early after infection and persists during disease progression,[22] leading to alterations of the DC/NK crosstalk.[6,23,24] In viremic HIV-1-infected patients, reduced NK-cell function is associated with low expression of NCRs[25] as well as with the expansion of the "anergic" $CD56^{-}/CD16^{+}$ ($CD56^{neg}$) NK-cell subset,[26] which is characterized by reduced NKp30 expression, decreased cytolytic functions and low cytokine production capacity.[26,27] The exhaustion of NK cells in chronic HIV-1-infected patients leads to altered DC editing, manifesting with an impaired killing of autologous immature DCs (iDCs). In particular, the markedly impaired expression and function of NKp30 among $CD56^{neg}$ NK cells subset largely accounts for the highly defective NK cell-mediated lysis of autologous iDCs.[6] In turn, mature DCs generated from HIV-1 viremic patients are substantially impaired in their ability to induce the proliferation of autologous NK cells, which consequently fail to secrete adequate amounts of interferon γ (IFNγ).[6] On the contrary, HIV-1-infected chimpanzees, which control infection when exposed to human-adapted HIV-1 variants, maintain functionally competent NK cells with high NCR expression during the course of infection,[7] confirming the importance of NCRs in this setting.

We have recently characterized NKp30 isoforms, demonstrating functional differences among the three major NKp30 splice variants: whereas NKp30a-transfected NKL cells (a human NK cell line)[28] block the proliferation of tumor cells harboring the NKp30 ligand B7-H6,[5] exhibit granule exocytosis into the microenvironment and kill tumor cells as well as iDCs, NKL cells that express NKp30b or NKp30c fail to do so (though the former preserve the capacity to respond to B7-H6-harboring cells by secreting T_H1 cytokines).[29] Most interestingly, in a retrospective analysis of 80 patients affected by gastrointestinal stromal tumor (GIST), a neoplasm that expresses NKp30 ligands and is sensitive to NK cell-mediated lysis, a predominant expression of the NKp30c isoform was associated with reduced patient survival, decreased NKp30-dependent tumor necrosis factor α (TNFα) and CD107a release, as well as defective IFNγ and interleukin (IL)-12 secretion in the NK-DC crosstalk, which could be restored by blocking IL-10. In line with this notion, the NKp30 status has been shown to predict the clinical outcome of patients with GIST.[29]

Considering (1) the critical role of NKp30 during NK-dependent DC maturation or killing and the subsequent polarization of immune responses, (2) the alteration of NKp30 and NKp46 expression on NK cells following HIV-1 infection and (3) the different functions of the three major isoforms of NKp30, we sought to determine the potential prognostic impact of the genetically determined NKp30 status on the control of HIV-1 infection in a historical cohort of HIV-1-infected untreated patients, the ANRS SEROCO.

Results

Expression of NKp30 and NKp46 receptors on peripheral blood NK cells. Peripheral blood mononuclear cells (PBMCs) from $HIV\text{-}1^{+}$ patients (n = 89) and healthy donors (HDs) (n = 10) were analyzed by flow cytometry to determine the relative abundance of NK-cell subsets as well as their expression levels of NKp30 and NKp46 (**Fig. 1A**). NK cells were identified as $CD3^{-}$, $TCR\gamma\delta^{-}$, $CD56^{+}$ and/or $CD16^{+}$ cells (**Fig. 1**). Among total NK cells, three subpopulations were defined based on the levels of expression of CD56 and CD16: $CD56^{bright}/CD16^{-}$ ($CD56^{bright}$), $CD56^{dim}/CD16^{+/-}$ ($CD56^{dim}$) and $CD56^{-}/CD16^{+}$ ($CD56^{neg}$). For each of these NK-cell subtypes, the percentage of $NKp30^{+}$ or $NKp46^{+}$ cells was evaluated together with the mean fluorescence intensity (MFI) of NKp30 or NKp46 expression on positive cells (**Fig. 1**).

We found no significant difference in the percentage of NK cells nor in the distribution of NK-cell subsets ($CD56^{bright}$, $CD56^{dim}$, $CD56^{neg}$) between $HIV\text{-}1^{+}$ patients and HDs (**Fig. S1**). In particular, the average percentage of $CD56^{neg}$ cells in HDs was 15.6% (6.0–26.7%) and in HIV^{+} patients 26.9% (4.0–77%). Nevertheless, we observed a decrease in the percentage of NK cells expressing NKp30 in $HIV\text{-}1^{+}$ patients as compared with HDs (p = 0.0011) (**Fig. 1B**), while the expression level of NKp30 on a per cell basis (evaluated by MFI on positive cells) remained stable (**Fig. 1B**). A reduction in the percentage of $NKp30^{+}$ cells was also observed among $CD56^{dim}$ and $CD56^{neg}$ cells of HIV-1-infected subjects compared with HD-derived cells (p = 0.085 and p = 0.0168, respectively) (**Fig. 1B**). Meanwhile, no significant differences were detected in the expression levels of the activating receptor NKp46, regardless of the NK-cell subset considered (**Fig. 1B**). Hence, HIV^{+} individuals exhibit a downregulation of NKp30 expression on peripheral NK cells.

NKp30 expression levels and clinical predictors. To analyze whether a reduced expression of NKp30 was related to transcriptional defects, we isolated total RNA from the PBMCs of 89 $HIV\text{-}1^{+}$ patients and 87 HDs, and quantified the expression levels of the three major NKp30 isoforms (a, b and c) using quantitative reverse transcription-PCR (qRT-PCR) and the ΔΔCt analysis method. The mean ΔCT ($Ct_{NKp30} - Ct_{β2M}$) values for NKp30a, NKp30b and NKp30c in the HD group were 9.69, 7.35 and 8.83, respectively.

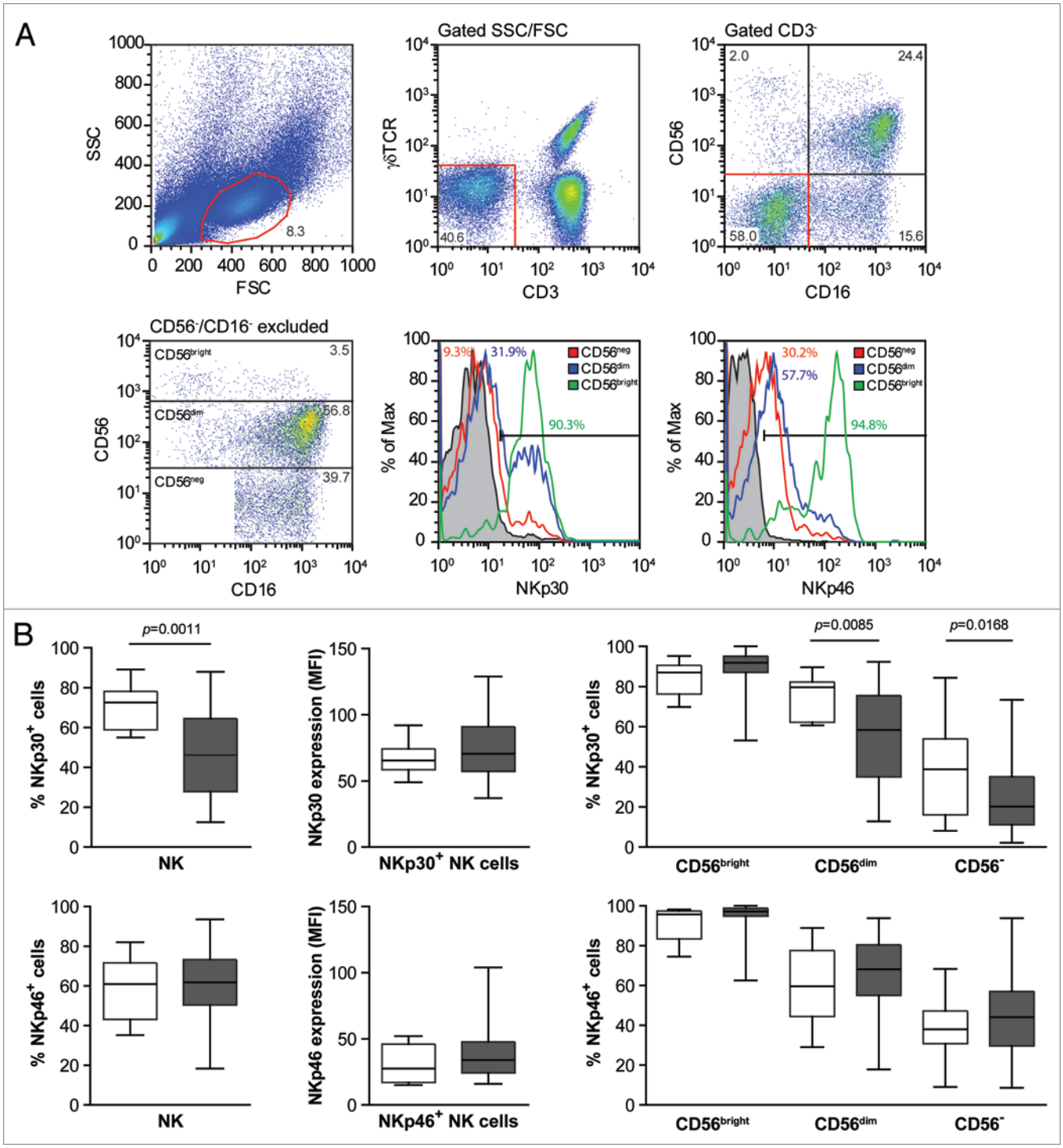

Figure 1. HIV-1+ seroconverters exhibit lower percentage of NK cells expressing NKp30 on their membrane, as compared with healthy donors. (**A and B**) Frozen peripheral blood mononuclear cells (PBMCs) isolated from HIV-1+ patients and healthy donors (HDs) were stained with CD3, CD16, CD56, γδTCR, NKp30 and NKp46-specific antibodies and analyzed by flow cytometry. (**A**) Gating procedure and NKp30 and NKp46 expression among natural killer (NK) cells of the indicated subsets (one representative experiment out of 89 is shown). (**B**) White and gray box plots represent data for HDs (n = 10) and HIV-1+ subjects (n = 74), respectively (middle bars = median values, box plots = 25% and 75% percentiles, whiskers = minimum and maximum values). Statistically significant p values are reported (unpaired, two-tailed Student's t-test).

By means of an unsupervised hierarchical clustering based on log-transformed and median-centered data, HIV-1+ patients were then clustered into three groups reflecting the mRNA expression level of the three NKp30 isoforms, compared with HDs (ΔΔCt cluster): high NKp30 (n = 20, ΔCT NKp30a: 9.66; NKp30b: 7.49; NKp30c: 8.95, comparable to the values of HDs), intermediate

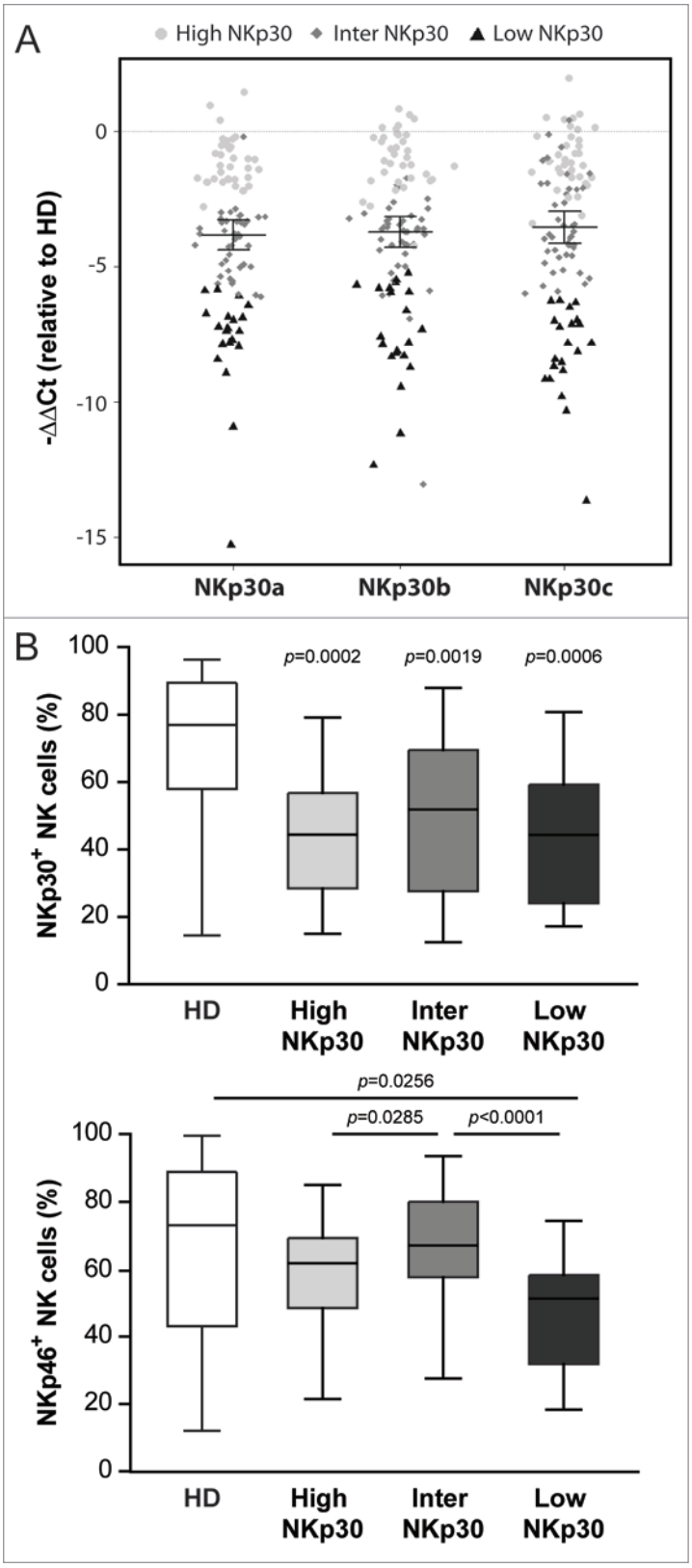

Figure 2. Seroconverters clustered based on *NCR3* mRNA expression levels do not differ in terms of relative abundance of NKp30$^+$ NK cells. (**A and B**) Total RNA was isolated from the peripheral blood mononuclear cells (PBMCs) of HIV-1$^+$ patients and healthy donors (HDs) and quantified by qRT-PCR. (**A**) HIV-1$^+$ patients were clustered into three groups based on *NCR3* expression levels as compared with the HD group (ΔΔCt cluster). The difference between NKp30 levels in HIV$^+$ patients and HDs is reported. (**B**) Percentage of NKp30$^+$ or NKp46$^+$ natural killer (NK) cells for each of the three patient groups as identified by the ΔΔCt cluster are shown. Middle bars = median values, box plots = 25% and 75% percentiles, whiskers = minimum and maximum values. Statistically significant p values are reported (unpaired, two-tailed Student's t-test). In the upper panel, p values refer to the difference between each group of HIV-1$^+$ subjects and HDs.

NKp30 (n = 42, ΔCT NKp30a: 12.36; NKp30b: 10.02; NKp30c: 10.95) and low NKp30 (n = 23, ΔCT NKp30a: 16.20; NKp30b: 13.46; NKp30c: 15.83) (**Fig. 2A; Table 1**). NKp30 mRNA expression level could not be determined in 4 patients.

For each group of patients defined by the ΔΔCt cluster, the percentage of NK cells expressing membrane NKp30 or NKp46 was determined by flow cytometry (**Fig. 2B**). Among the three ΔΔCt cluster groups, the percentages of NKp30$^+$ NK cells were comparable (**Fig. 2B**, upper panel), although significant differences were found in the percentage of NKp46$^+$ NK cells (**Fig. 2B**, lower panel), suggesting that the reduced expression of NKp30 on the surface of NK cells from HIV$^+$ individuals does not result from transcriptional alterations of the three main NKp30 isoforms.

We then addressed whether the mRNA expression levels of *NCR3* may influence the progression of HIV-1 infection. For each of the three ΔΔCt cluster groups, we evaluated the time- to-CD4$^+$ T-cell loss (based on the number of patients whose CD4$^+$ cell count fell below 200 cells/mm^3 at two consecutive visits) (**Fig. 3A**), the time-to-first AIDS-defining illness (**Fig. 3B**) and survival (**Fig. 3C**). We observed no association between *NCR3* mRNA levels in the three ΔΔCt cluster groups and these parameters (p = 0.89, p = 0.93, p = 0.54, for CD4$^+$ T-cell count fall, AIDS and survival, respectively).

Relative NKp30 isoform expression levels and clinical predictors. The levels of expression of the three major NKp30 isoforms were measured by qRT-PCR using RNA extracted from PBMCs, purified total NK cells, CD56^{bright}, CD56^{dim} and CD56^{neg} NK-cell subsets from 10 HIV-1$^+$ patients. The relative expression of the different isoforms compared with each other was calculated using the "ratio" formula: $NKp30_x$ / $NKp30_y$ = 2^(ΔΔCt $NKp30_y$ – ΔΔCt $NKp30_x$). The relative expression of NKp30 isoforms was similar in all cell subsets analyzed (**Fig. S2**). Furthermore, the NKp30 isoform profile was stable over time, as shown by a longitudinal analysis performed in the 10 HIV-1$^+$ patients at two time points with a mean temporal distance of 5.5 y (**Fig. S3**).

Unsupervised hierarchical clustering was subsequently performed on the relative NKp30 isoform expression data from 56 HDs and 89 HIV-1$^+$ patients. The A vs. B, B vs. C and A vs. C distribution on HDs is shown in **Figure S4**. The clustering of HIV$^+$ subjects resulted in the definition of three groups of patients with distinct NKp30 profile (ratio cluster): patients presenting as the most remarkable feature a low expression level of the c isoform (Low C, n = 40), a high expression level of the b isoform (High B, n = 15) and a high expression level of

Table 1. Patients' characteristics and ΔΔCt cluster

Characteristics	Total (n = 89)	#NKp30^{High} (n = 20)	#NKp30^{Int} (n = 42)	#NKp30^{Low} (n = 23)	Statistics	
Sex*					p^1	
Men	67 (75%)	15 (75%)	31 (74%)	17 (74%)	**1**	
Women	22 (25%)	5 (25%)	11 (26%)	6 (26%)		
Mode of transmission*						
Men from Men	31 (69%)	13 (65%)	27 (64%)	17 (74%)		
Men from Women	6 (7%)	2 (10%)	4 (10%)	0 (0%)	**0. 69**	
Women from Men	22 (25%)	5 (25%)	11 (26%)	6 (26%)		
Time (in months) from infection to **					p^2	
Inclusion	6.2 [1.3–43.5]	5.4 [2.7–41.2]	8.9 [1.3–43.5]	6.1 [2.1–10.1]	**0.08**	
NKp30 study	38.3 [3.6–44.4]	40.0 [8.5–44.0]	28.0 [3.6–44.4]	41.8 [17.2–44.4]	**0.008**	
***Biological markers** at inclusion*					p^2	p^3
CD4$^+$ cells	556 [72–1330]	458.5 [268–1260]	542 [72–1193]	616 [224–1330]	**0.05**	**0.09**[a]
Log(HIV-1 RNA)	4.1 [2.4–5.6]	4.3 [3.5–5.6]	4.1 [3.1–5.3]	3.9 [2.4–5.5]	**0.18**	**0.18**[a]
Log(HIV-1 DNA)	2.8 [1.7–4.0]	2.8 [1.8–3.6]	2.7 [1.7–3.7]	2.9 [1.7–4.0]	**0.57**	**0.60**[a]
***Biological markers** at* **NKp30 status assessment**						
CD4$^+$ cells	478 [19–1193]	479 [244–797]	490 [72–1193]	456 [19–917]	**0.60**	**0.62**[b]
Log(HIV-1 RNA)	4.0 [2.4–5.6]	3.7 [3.0–5.5]	4.0 [3.0–5.1]	4.0 [2.7–5.6]	**0.99**	**0.99**[b]
Log(HIV-1 DNA)	2.9 [1.6–5.2]	3.0 [2.0–5.2]	2.9 [1.9–3.7]	3.0 [1.6–4.0]	**0.75**	**0.45**[b]

#ΔΔCt cluster groups; *number (%); **median [min-max]; [1]Fisher's exact test; [2]Kruskal-Wallis rank sum test; [3]Linear regression adjusted for: [a]mean time from contamination to inclusion in the study, [b]mean time from contamination to NKp30 status assessment.

the c isoform (High C n = 28) (**Fig. 4A; Table 2**). The NKp30 isoform profile could not be determined in 6 patients.

In each of these three groups, we assessed the percentage of NKp30$^+$ or NKp46$^+$ NK cells (by flow cytometry). The three groups of patients showed a decrease in the percentage of NKp30$^+$ NK cells as compared with HDs ($p < 0.0001$, $p = 0.0064$ and $p = 0.0031$ for the Low C, High B and High C groups, respectively) but no difference was detected in the percentage of NKp30$^+$ or NKp46$^+$ NK cells between the three ratio cluster groups (**Fig. 4B**).

We finally searched for a potential influence of NKp30 isoform expression profile on disease evolution. The Kaplan-Meier curves shown in **Figure 5** illustrate that there is no association between the NKp30 isoform profile and time-to-CD4+ T-cell loss ($p = 0.58$), time-to-clinical AIDS ($p = 0.64$) or survival ($p = 0.59$).

Frequency of CD56^{neg} NK cells and prognostic factors. As mentioned above, we found no significant expansion of the CD56^{neg} NK-cell subset in HIV-1$^+$ patients as compared with HDs (**Fig. S1**). Nevertheless, we evaluated whether the percentage of CD56^{neg} NK cells would be correlated with the CD4$^+$ cell count, plasma viral load or cell-associated proviral DNA at the time of our study. No association was found between the proportion of CD56^{neg} NK cells and these parameters (**Fig. 6**) among the 89 patients included in the study (on average 6 mo post-infection), with a median serum viral load of 12,589 HIV-1 RNA copies/mL and a CD4$^+$ cell count of 556 cells/mm^3 (**Table 1**).

Discussion

The aim of our study was to assess the potential role of NKp30-related parameters (NKp30 surface expression, NKp30 transcriptional levels, NKp30 isoforms) on the progression of HIV-1 infection. The finding that the preferential expression of the immunosuppressive NKp30c isoform is associated with poor prognosis in GIST patients[29] prompted us to perform a retrospective analysis of 89 HIV-1-infected individuals from the ANRS CO 02 SEROCO-HEMOCO cohort. This cohort included patients enrolled early after HIV seroconversion and followed from 1988 to 1995, before the introduction of highly active antiretroviral therapy (HAART). Thus, this cohort offered an extended follow-up of untreated patients, allowing us to determine the influence of the NKp30 isoform profile on spontaneous disease progression. We found that neither NKp30 expression levels nor the NKp30 isoform profile correlates with the virological and clinical parameters analyzed in this cohort of patients for whom the natural history of HIV-1 infection is available. Nevertheless, we noticed that—at the time of inclusion in the cohort—individuals belonging to the Low B group (exhibiting a reduced expression of NKp30b) were characterized by a significantly higher CD4$^+$ T-cell count and a lower viral load as compared with the other groups of patients (**Table 2**). This might suggest an early effect of the NKp30 profile on the loss of CD4$^+$ cells, which may become irrelevant with the progression of disease. Further studies are needed to test this hypothesis on a more homogeneous cohort of acutely infected patients.

Comparative flow cytometry analyses of NK-cell subsets on frozen PBMCs from HIV-1$^+$ patients and HDs showed no difference in the percentage of NK cells among PBMCs nor in the proportion of CD56^{bright}, CD56^{dim} and CD56^{neg} cells among NK cells (**Fig. S1**). This is in contrast with previous observations by Mavilio et al., who reported that in viremic patients with chronic

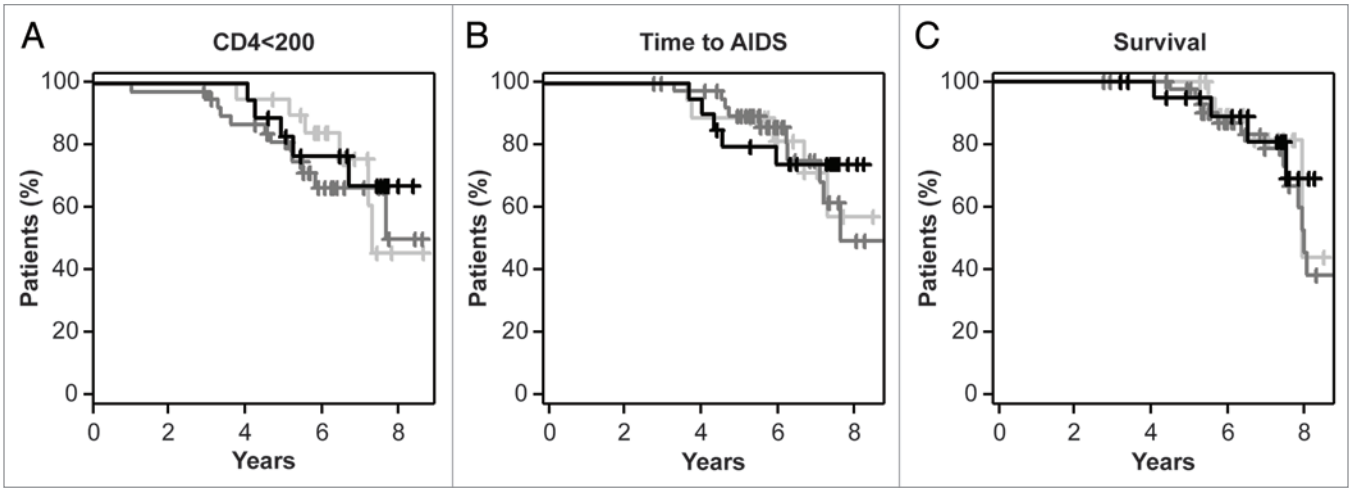

Figure 3. *NCR3* mRNA expression levels neither correlate with the loss of CD4⁺ T cells nor constitute a prognostic factor for time-to-AIDS or overall survival. For each of the three groups of patients as identified by the ΔΔCt cluster (light gray = High NKp30, dark gray = Inter NKp30, black = Low NKp30), Kaplan-Meier curves for time-to-loss of CD4⁺ T cells (< 200/mm³) time-to-first AIDS-defining event and survival are shown.

HIV-1 infection, the proportion of $CD56^{dim}$ NK cells is sharply decreased and the $CD56^{neg}$ NK cells subpopulation is expanded as compared with HDs.[30] This apparent discrepancy may be due to the differences in disease stage, i.e., recent infection vs. chronic late infection, across the two groups of patients studied, suggesting that alterations in the representation of NK-cell subsets require several years of infection to occur.

In HIV-1 infected individuals, we observed a decrease in the percentage of NK cells expressing NKp30, affecting both $CD56^{dim}$ and $CD56^{neg}$ NK cells (**Fig. 1B**). In line with this observation, Mavilio et al. reported that viremic, but not aviremic, chronically infected patients exhibit a significant downregulation of NCRs, including NKp30 and NKp46.[30] In contrast, we observed no difference in the expression of NKp46 between HIV-1⁺ patients and HDs (**Fig. 1B**), suggesting that alterations in NKp46 expression appears later after primary infection. We did not find any association between the percentage of NKp30⁺ NK cells in HIV⁺ patients and the clinical parameters that we evaluated (loss of CD4⁺ T cells, time-to-AIDS-defining event or patient survival) (data not shown).

We subsequently quantified *NCR3* mRNA from the PBMCs of both seroconverters and HDs, normalizing NKp30 expression levels to those of the housekeeping gene β2 microglobulin (*B2M*). The unsupervised hierarchical clustering of *NCR3* mRNA expression data (ΔΔCt clustering) allowed us to classify patients into three groups expressing high, intermediate and low levels of NKp30 (**Fig. 2A**). The High NKp30 group showed NKp30 levels comparable to those observed among HDs (**Fig. 2A**). Interestingly, no correlation between NKp30 mRNA levels (ΔΔCt cluster groups) and surface NKp30 expression levels (assessed by flow cytometry) was found, in terms of both percentage of NKp30⁺ cells (**Fig. 2B**) and MFI on NKp30⁺ cells (data not shown), perhaps suggesting a consistent degree of post-transcriptional regulation of NKp30.

We then addressed the question as to whether the NKp30 isoform profile may influence the progression of HIV-1 infection. We performed qRT-PCR using primers specific for each of the three major NKp30 isoforms (NKp30a, NKp30b and NKp30c) and their relative expression level was calculated for all patients. These expression levels were comparable whether evaluated on the RNA from PBMCs or from purified NK cells, $CD56^{bright}$, $CD56^{dim}$ or $CD56^{neg}$ cells (**Fig. S2**). NKp30 isoform profiles in HIV-1⁺ subjects were found to be stable over time (**Fig. S3**), similar to what has previously been shown for GIST patients,[29] allowing us to analyze a single time point per patient. The unsupervised hierarchical clustering of the relative expression levels of the three isoforms (ratio clustering) resulted in the classification of patients into three groups: Low C, High B and High C (bearing low levels of the c isoform or high levels of the b or c isoforms, respectively) (**Fig. 4A**). These three groups of patients did not differ in terms of percentage of NKp30⁺ NK cells (**Fig. 4B**), nor in terms of NKp30 expression level on NKp30⁺ cells (data not shown). Therefore, our analysis of the influence of the NKp30 isoform profile on HIV-1 disease progression is unlikely to be biased by differences in surface expression levels of NKp30, yet suggest no prognostic significance for this parameters (at least in our cohort).

We have previously reported that the activation of NK cells bearing different NKp30 isoforms results in different functional outcomes. The predominant expression of the immunosuppressive NKp30c isoform has indeed been associated with reduced survival of GIST patients, correlating with defective IFNγ, TNFα and IL-12 production in the NK-DC crosstalk, which could be restored by blocking IL-10.[29] In spite of the important role played by NK cells during both acute and chronic HIV-1 infection,[22] the crucial function of NKp30 in NK-cell activity,[6,25] and the multiple effects exerted by IL-10 during HIV-1 infection,[31] we were not able to detect in our cohort of 89 recently seroconverted HIV-1⁺ patients any association between the *NCR3* mRNA expression levels or NKp30 isoform profiles and the clinical parameters that we evaluated, i.e., the loss of CD4⁺ T cells, the time-to-clinical AIDS and survival (**Figs. 3 and 5**).

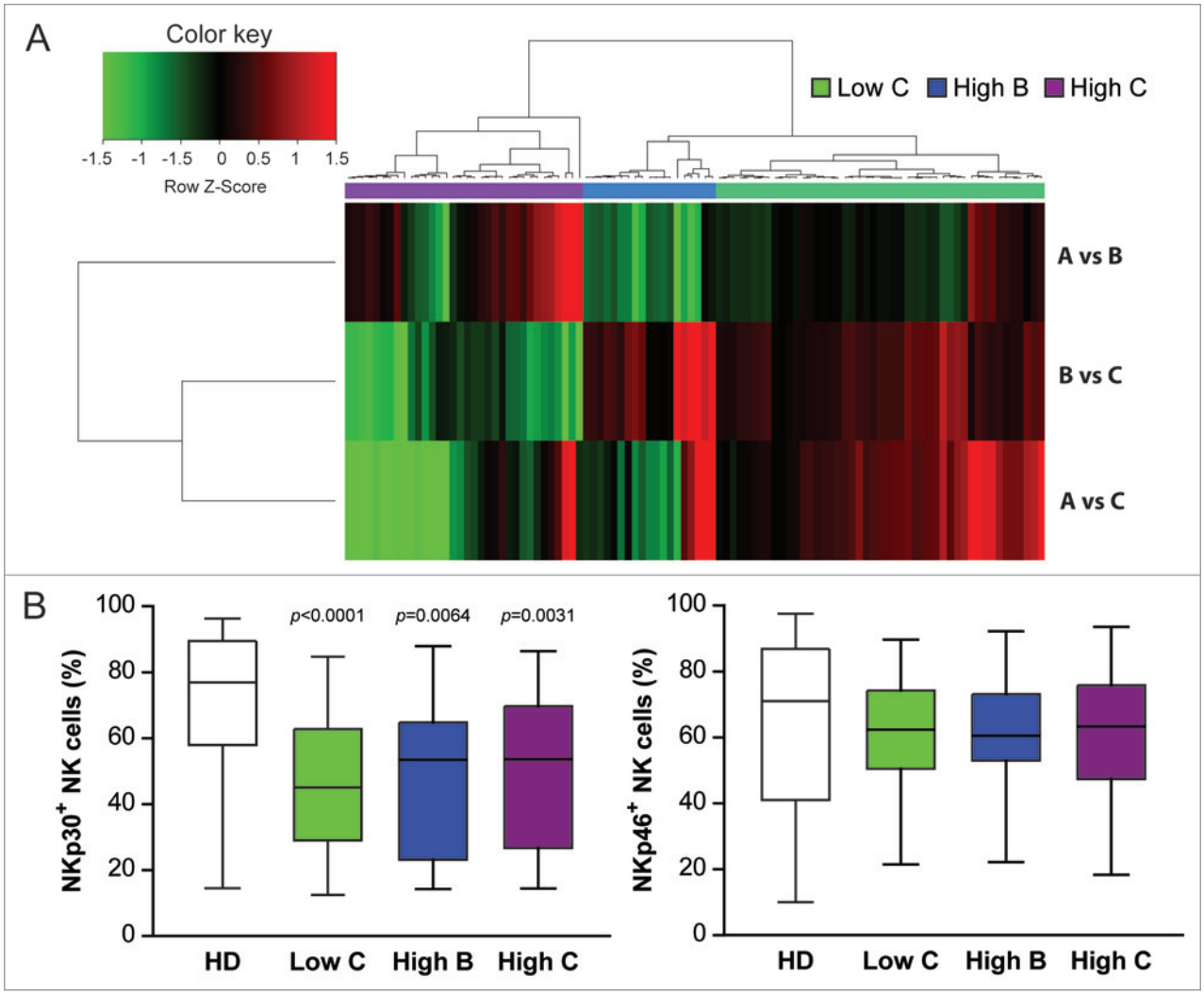

Figure 4. Patients clustered based on their NKp30 isoform profile do not differ in terms of percentage of NKp30$^+$ NK cells. (**A and B**) Total RNA was isolated from the peripheral blood mononuclear cells (PBMCs) of HIV-1$^+$ patients and healthy donors (HDs) and quantified by qRT-PCR. (**A**) HIV-1$^+$ patients were clustered in three groups based on the relative expression levels of the three major NKp30 isoforms (ratio cluster). (**B**) Percentage of NKp30$^+$ or NKp46$^+$ natural killer (NK) cells for each of the three ratio cluster groups. Boxes = 25% and 75% percentiles, middle bars = median values; whiskers = minimum and maximum values. Statistically significant p values are reported (unpaired, two-tailed Student's t-test). In the left panel, p values refer to the difference between each of the three groups of HIV-1$^+$ subjects and HD.

Finally, we did not find any correlation between the percentage of CD56^{neg} NK cells and CD4$^+$ T-cell count, plasma viral load or proviral DNA levels (**Fig. 6**), in contrast with a previous report showing an association between the expansion of the CD56^{neg} subpopulation and viremia.[26] However, our study involves patients at an earlier stage of infection, leading us to speculate that alterations in the NK-cell subsets distribution, notably the expansion of the CD56^{neg} subpopulation, are linked to persistent viral replication and hence constitute a late consequence of immune dysfunction.

Altogether, our observations do not support any correlation between NKp30 status and the clinical outcome of recently infected HIV-1$^+$ patients that were left untreated for more than 3 y. However, we must acknowledge some potential limitations that might have undermined our study. First, although this cohort allowed us to follow the natural evolution of HIV-1 infection, the number of samples per patient was restricted. Second, given the general heterogeneity of HIV-1 infected patients, a greater number of patients may be needed to detect a correlation between NKp30 status and clinical outcome. Finally, due to the limited number of patient analyzed, we could not establish whether the differences among ratio cluster groups in CD4$^+$ T-cell count and viremia at the time of inclusion (**Table 2**) have a biological meaning or reflect biases that may have compromised our analysis. It is time to reevaluate the influence of NKp30 status on the evolution of HIV-1 infection in the setting of primary infection or in long-term non-progressors.

Materials and Methods

Study population. Frozen PBMCs were obtained from patients enrolled in the ANRS CO 02 SEROCO-HEMOCO cohort,

Table 2. Patients' characteristics and ratio cluster

Characteristics	#Low C (n = 40)	#Low B (n = 15)	#High C (n = 28)	Statistics	
Sex*				p^1	
Men	30 (75%)	11 (73%)	22 (79%)	**0.89**	
Women	10 (25%)	4 (27%)	6 (21%)		
Mode of transmission*					
Men from Men	27 (68%)	11 (73%)	19 (68%)		
Men from Women	3 (8%)	0 (0%)	3 (11%)	**0.87**	
Women from Men	10 (25%)	4 (27%)	6 (21%)		
Time (in months) from infection to **				p^2	
Inclusion	6.0 [1.3–43.5]	6.8 [1.8–35.5]	6.6 [1.3–36.5]	**0.72**	
NKp30 study	38.7 [4.7–44.3]	35.5 [10–44]	39.2 [3.6–44.4]	**0.75**	
Biological markers** at inclusion				p^2	p^3
$CD4^+$ cells	485.5 [202–987]	874 [415–1330]	542 [72–1193]	**< 0.01**	**< 0.01[a]**
Log(HIV-1 RNA)	4.2 [3.4–5.6]	3.5 [2.4–4.9]	4.1 [2.9–5.5]	**0.06**	**0.02[a]**
Log(HIV-1 DNA)	3 [1.8–4]	2.6 [1.7–3.2]	2.7 [1.7–3.5]	**0.17**	**0.16[a]**
Biological markers** at NKp30 status assessment					
$CD4^+$ cells	457.5 [19–987]	578 [305–940]	488 [72–1193]	**0.16**	**0.09[b]**
Log(HIV-1 RNA)	4 [3–5.6]	3.7 [3.1–5]	4 [2.7–5.4]	**0.62**	**0.33[b]**
Log(HIV-1 DNA)	3.2 [1.6–5.2]	2.6 [1.7–3.5]	2.9 [1.9–3.7]	**0.14**	**0.10[b]**

#Ratio cluster groups; *number (%); ** median [min-max]; [1]Fisher's exact test; [2]Kruskal-Wallis rank sum test; [3]Linear regression adjusted for: [a]mean time from contamination to inclusion in the study, [b]mean time from contamination to NKp30 status assessment.

which includes individuals with a recent seroconversion or recent HIV-1 diagnosis enrolled from 1988 to 1995.[32] Among these patients, we selected 89 individuals for whom frozen cells were available, who did not present hepatitis B virus or hepatitis C virus co-infection, who were not intravenous drugs users, hemophiliac, or pregnant, had neither autoimmune diseases nor malignancies within the 5 y preceding their enrolment, had no concomitant or previous treatment with interferon and other cytokines, steroids or other immunomodulators. The characteristics of these patients are reported in **Table 1**. Ten HDs served as controls for immunological parameters. A written informed consent was obtained from patients, in line with the guidelines formulated by local ethical committees.

qRT-PCR. The levels of expression of the three major NKp30 isoforms were measured by qRT-PCR and normalized to the level of expression of the housekeeping gene β-2-microglobulin (*B2M*), as previously described.[29] Total cellular RNA was isolated, by means of the RNeasy Mini kit (Qiagen, 74106), from frozen PBMCs, purified NK cells (isolated from PBMCs by magnetic sorting using the EasySep Human NK Cell Enrichment Kit, from Stem Cell, 19055) or purified NK-cell subpopulations (isolated from total NK cells using the a FACSAria cell sorter, from BD Biosciences). cDNA was synthesized from total RNA using the SuperScript™ III Reverse Transcriptase (Invitrogen, 18080-044) and random primers (Promega, C1181), according to the manufacturer's instructions. The following primers and probes (Applied Biosystems) were used for qRT-PCR: NKp30-EC (Fwd): 5'-TTT CCT CCA TGA CCA CCA GG-3'; NKp30-EX4I (Rev): 5'-TTC CCA TGT GAC AGT GGC ATT-3'; NKp30-EX4II (Rev): 5'-CGG AGA GAG TAG ATT TGG CAT ATT-3'; NKP30-EX4III (Rev): 5'-GGA CCT TTC CAG GTC AGA CAT T-3'; NKp30-Probe (6-FAM/TAMRA): 5'-TGG TGG AGA AAG AAC ATC CTC AGC TAG GG-3'; B2M-F (Fwd): 5'- GAT GAG TAT GCC TGC CGT GT-3'; B2M-R (Rev): 5'-AAT TCA TCC AAT CCA AAT GCG-3'; B2M-Probe (6-FAM/TAMRA): 5'-AAC CAT GTG ACT TTG TCA CAG CCC AA-3'. First-strand cDNA was amplified using TaqMan Gene Expression Master Mix (Applied Biosystems, 4369016) and NKp30 or B2M primers (10 μM) and probes (5 μM) in a final volume of 25 μL. One initial incubation at 50°C for 2 min was followed by one cycle of denaturation (95°C for 10 min) and 45 cycles of amplification (95°C for 15 sec and 60°C for 1 min). qRT-PCR was performed in a StepOnePlus System (Applied Biosystems), samples were amplified in triplicate and the qRT-PCR data were analyzed using the $2^{-\Delta Ct}$ method.

Unsupervised hierarchical clustering. The level of expression of NKp30 isoforms in HIV-1^+ patients compared with HDs was determined using the ΔΔCt method: $-\Delta\Delta CT = -[(\text{HIV-1}_{NKp30} - \text{HIV-1}_{B2M}) - (\text{HD}_{NKp30} - \text{HD}_{B2M})]$. The level of expression of the distinct NKp30 isoforms compared with each other in each patient (ratio) was determined using the following formula: $NKp30_x / NKp30_y = 2\text{^}(\Delta\Delta Ct\, NKp30_y - \Delta\Delta Ct\, NKp30_x)$. Unsupervised hierarchical clustering was applied to log-transformed and median-centered data using the Cluster and TreeView programs (average linkage clustering using Pearson's centered correlation as similarity metric). Two clusters were created: the first one based on the different levels of expression of NKp30 in HIV-1^+ patients compared with HDs (ΔΔCt cluster)

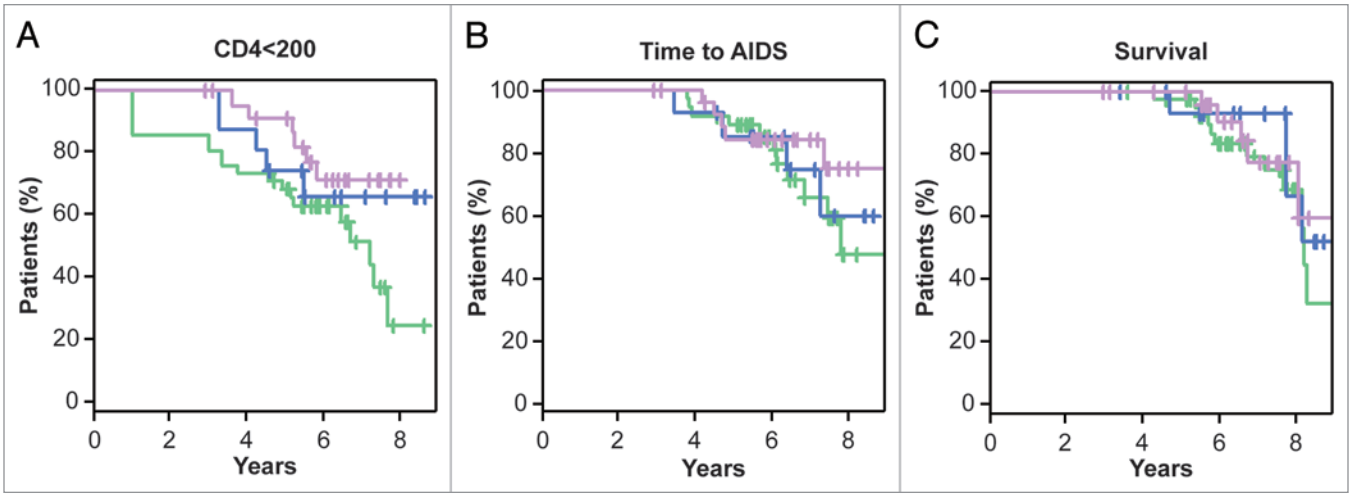

Figure 5. NKp30 isoform profiles do not correlate with the loss of CD4⁺ T cells and are not a prognostic factor for time-to-AIDS or survival. For each of the three groups of patients as identified by the ratio cluster (green = Low C, blue = High B, violet = High C), Kaplan-Meier curves for time-to-loss of CD4⁺ T cells (< 200/mm³), time-to-first AIDS-defining event and survival are shown.

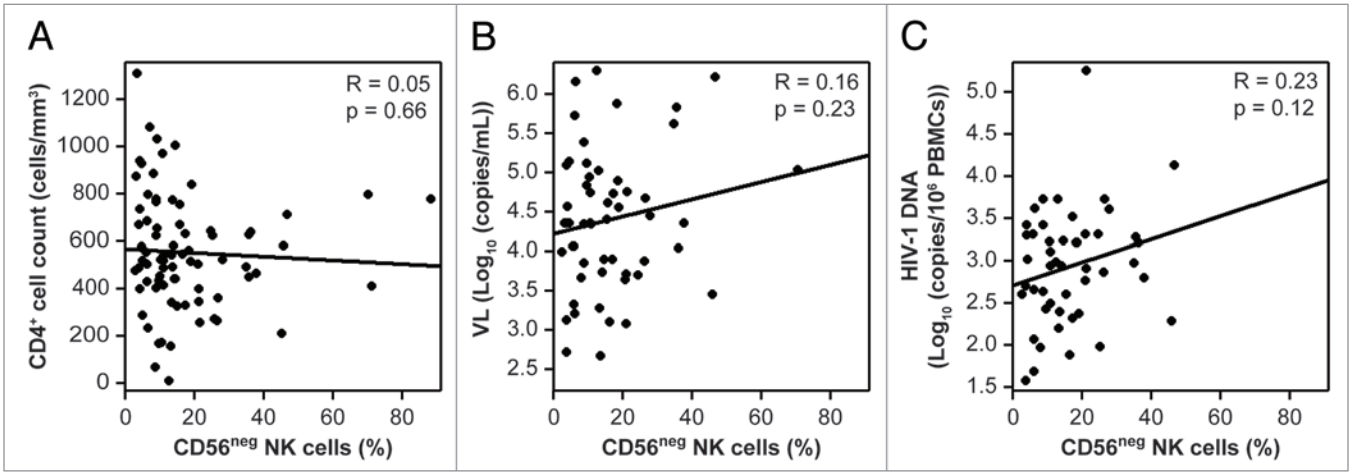

Figure 6. Lack of correlation between the percentages of CD56^{neg} NK cells and clinical parameters. (**A and C**) Percentage of CD56^{neg} cells among natural killer (NK) cells as a function of CD4⁺ T-cell counts (**A**), viral load (**B**) and proviral DNA levels (**C**) (Spearman correlation).

and the second based on the levels of expression of each NKp30 isoform as compared with the others in HIV-1⁺ patients (ratio cluster).

Flow cytometry. The following mouse anti-human fluorescent monoclonal antibodies were used: CD3-APC-Cya7 (Beckman Coulter, A94680), CD16-Pacific Blue (Beckman Coulter, A82792), CD56-PE-Cy7a (Beckman Coulter, A21692), γδTCR-FITC (Beckman Coulter), NKp30-PE (clone AF29-4D12) (Miltenyi, 130-092-483), NKp46-APC (Miltenyi, 130-092-609). In addition, the dead-cell removal reagent LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen, L34959) was employed. Frozen cells were thawed, washed and stained with the abovementioned reagents for 15 min at 4°C, washed and fixed with 1% PFA. Cells were acquired on a FACSAria cell sorter immediately following staining and analyses were performed using the FlowJo software (Tree Star).

Statistical analyses. The Fisher's exact test and the non-parametric Kruskal-Wallis rank sum test were used for the comparison of different groups. Survival curves were plotted according to the Kaplan-Meier method. A Cox model was employed to take into account time from infection to NKp30 status assessment (left-entry model) and was used to compare the survival according to NKp30 status. All analyses were performed with the R package version 2.14.2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be downloaded here:
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NCR3/NKp30 Contributes to Pathogenesis in Primary Sjögren's Syndrome

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Editor's Summary

Sjögren's Research to Make Your Mouth Water

Sjögren's syndrome is an autoimmune disorder where the body's own immune cells attack and destroy the exocrine glands that produce such things as tears and saliva. Some patients may have only minor irritation, whereas others may have more serious systemic effects. Sjögren's syndrome is most common in women over 40, and treatment only attempts to alleviate the symptoms —there is no cure. Now, Rusakiewicz *et al.* implicate natural killer (NK) cells in the pathogenesis of Sjögren's syndrome.

The authors found that a genetic polymorphism is NKp30, an NK cell–activating receptor, associated with susceptibility to Sjögren's syndrome in human patients compared with healthy controls. NK cells in these patients expressed high levels of NKp30 and secreted more proinflammatory cytokines. What's more, these NK cells accumulated in inflammatory foci in minor salivary glands, and salivary epithelial cells expressed B7H6, a ligand that activates NKp30. These data strongly suggest that NK cells may contribute to Sjögren's syndrome pathogenesis, and put forth NKp30 as a therapeutic target, providing a potential oasis for Sjögren's patients.

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SJÖGREN'S SYNDROME

NCR3/NKp30 Contributes to Pathogenesis in Primary Sjögren's Syndrome

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Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterized by a lymphocytic exocrinopathy. However, patients often have evidence of systemic autoimmunity, and they are at markedly increased risk for the development of non- Hodgkin's lymphoma. Similar to other autoimmune disorders, a strong interferon (IFN) signature is present among subsets of pSS patients, although the precise etiology remains uncertain. NCR3/NKp30 is a natural killer (NK)–specific activating receptor regulating the cross talk between NK and dendritic cells and type II IFN secretion. We performed a case-control study of genetic polymorphisms of the NCR3/NKp30 gene and found that rs11575837 (G>A) residing in the promoter was associated with reduced gene transcription and function as well as protection to pSS. We also demonstrated that circulating levels of NCR3/NKp30 were significantly increased among pSS patients compared with controls and correlated with higher NCR3/NKp30 but not CD16-dependent IFN-γ secretion by NK cells. Excess accumulation of NK cells in minor salivary glands correlated with the severity of the exocrinopathy. B7H6, the ligand of NKp30, was expressed by salivary epithelial cells. These findings suggest that NK cells may promote an NKp30-dependent inflammatory state in salivary glands and that blockade of the B7H6/NKp30 axis could be clinically relevant in pSS.

INTRODUCTION

Although primary Sjögren's syndrome (pSS) affects up to 0.1 to 0.6% of the general population, few rigorous etiologic studies have focused on the initiating immunopathologic process occurring in the exocrine glands. Histologic examination of minor salivary glands (MSGs) obtained from pSS patients reveals persistent inflammatory foci consisting of mononuclear cells [primarily T lymphocytes, with some B lymphocytes and rare dendritic cells (DCs) (*1*)], leading to progressive acinar epithelial cell atrophy and fibrosis. The severity of secretory dysfunction does not always correlate with the extent or degree of leukocytic infiltration and loss of acinar tissue, suggesting that immune dysregulation might impair exocrine gland secretion very early in the disease process (*2*, *3*).

Gene expression profiling studies demonstrated activation of type I and II interferon (IFN) pathways in pSS patients (*4*–*7*). B cells are highly activated in this autoimmune disease through diverse pathways, including the overexpression of the BAFF cytokine (*8*), and T cell patterns are skewed toward a memory phenotype and a T helper 1 (T_H1) polarization associated with high serum levels of IFN-γ (*9*). Recently, elegant studies reported data supporting the potential role of innate or cognate (double negative or conventional T cells) effectors in setting the stage of a T_H17-based inflammation in pSS (*10*–*13*).

Animal models of pSS have provided insight into both genetic susceptibility and key pathologic molecules participating in the onset (preclinical phase) or development (clinical phase) of pSS (*14*). For example, studies performed in NOD.IFN-$\gamma^{-/-}$ or NOD.IFN-$\gamma R^{-/-}$ mice indicate that IFN-γ is a crucial cytokine for pSS-like disease (*15*). More specifically, NOD.IFN-$\gamma^{-/-}$ and NOD.IFN-$\gamma R^{-/-}$ mice fail to develop autoimmune responses, acinar cell apoptosis, and leukocyte infiltration against salivary glands at the clinical stage of the disease (*15*). IFN-γ is central to salivary gland dysfunction in the Ro60 humanized model of Sjögren's syndrome (*16*). Recently, use of precise probes distinguishing type I from type II activity showed that the pSS-associated IFN signature could be assigned, at least in some cases, to IFN-γ (*6*). Moreover, interleukin-12 (IL-12) (a strong IFN-γ inducer) transgenic mice develop a pSS-like syndrome (*17*, *18*). Last, a genetic polymorphism of IL-12A was recently shown to be associated with pSS in humans based on a genome-wide association study (*19*), and our group recently showed that IL-12 can induce IFN-γ as well as IFN-α secretion in human pSS (*20*).

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As a result of their interaction with various DC subsets, natural killer (NK) cells play a critical role as mediators of both type I and type II IFN biologic functions. Although animal models of pSS have not directly implicated NK cells in disease pathogenesis (*21*), recent work in mouse cytomegalovirus-induced sialadenitis, which may be highly relevant to pSS, implicates a regulatory role for salivary NK cells (*22*). Flow cytometry analyses of salivary glands from pSS patients demonstrate the presence of a subset of NK cells expressing NKp44 and producing IL-22, which are associated with the severity of sialadenitis (*11*).

The aim of this study was to investigate the role of NK cells in pSS pathogenesis. We focused on *NCR3*/NKp30 effector functions for several reasons. First, NKp30 is selectively expressed by circulating NK cells and represents a surrogate marker of NK cell functions in humans (*23*). Second, NKp30 is a crucial regulator of the DC/NK cell cross talk, mediating the release of T_H1 (IL-12 and IFN-γ) cytokines (*24*). Third, transcription of the *NCR3*/NKp30 gene is subject to alternative splicing, resulting in various isoforms endowed with distinct effector functions (*25*). Here, we show that genetic polymorphisms within the promoter region of *NCR3*/NKp30 were associated with reduced levels of gene transcription and function as well as decreased susceptibility to pSS. Thus, NKp30 expression and functions were found to be up-regulated in pSS (compared with controls). NK cells correlate with the sialadenitis, whereas salivary gland epithelial cells could express NKp30 ligands and trigger NKp30-dependent release of T_H1 cytokines.

RESULTS

Genetic polymorphisms within the NKp30 promoter region associated with decreased susceptibility to pSS

Previous work has defined three isoforms of NKp30 with distinct effector functions that have prognostic relevance in gastrointestinal stromal tumor malignancies (*25*). The NKp30 A and B isoforms, having high killing activity and secretory capacity for IFN-γ and TNF-α (tumor necrosis factor–α), respectively, are associated with a better prognosis than NKp30 isoform C. Thus, we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) using primers specific for NKp30A, NKp30B, or NKp30C in 164 pSS patients from the French Assessment of Systemic Symptoms and Evolution in patients with pSS (ASSESS) cohort and compared the relative amounts of each isoform by calculating ΔC_t ratios (A/B, B/C, and A/C). The unsupervised hierarchical clustering of expression data defined six distinct groups (fig. S1) that did not appear to be associated with specific pSS phenotypes, such as systemic involvement, presence of autoantibodies, or history of lymphoma.

Then, we studied single-nucleotide polymorphisms (SNPs) that could affect the transcription of the NKp30 gene. In the exploratory cohort, 574 French pSS patients and 451 independent French control subjects were genotyped for nine SNPs within the 6p21.3 NKp30 locus (table S1). Because of the proximity of *NKp30* to the HLA-DR (human leukocyte antigen–DR) and TNF loci, which have previously been implicated in pSS (*26*, *27*), three additional SNP proxies for HLA-DR2, HLA-DR3, and TNF-308 were investigated. Single-SNP analyses adjusting for ancestry (see Materials and Methods) showed that four SNPs within the *NKp30* locus, including rs2736191 and rs11575837, were inversely associated with risk of pSS (table S2). These associations were neither stronger nor more statistically significant among subsets of patients with disease-specific autoantibodies (table S2).

Two *NKp30* SNPs (rs11575837 and rs2736191) and the SNP proxy for *HLA-DR3* (rs2187668) were genotyped in the replication study that included 436 pSS patients from Sweden ($n = 244$) and Norway ($n = 192$) and 441 healthy controls (Sweden, $n = 232$; Norway, $n = 209$). In the replication study, the *NKp30* variant rs11575837 was significantly associated with disease characterized by anti-SSA and/or anti-SSB positivity [$P = 0.011$, odds ratio (OR) = 0.060] (Table 1).

Table 1. Single-SNP logistic regression results adjusted for top two PCs, by cohort and combined via meta-analysis: (A) pSS cases versus healthy controls, (B) SSA/B+ pSS cases versus controls, and (C) SSA/B+ pSS cases versus SSA/B– pSS cases. SSA/B+ cases are SSA-positive or SSB-positive. SSA/B– cases are SSA-negative and SSB-negative. MAF, minor allele frequency.

		Exploratory (French) cohort				Replication (Scandinavian) cohort				Meta-analysis of two cohorts				
		574 pSS cases (340 SSA/B+, 233 SSA/B–) and 451 controls				436 pSS cases (329 SSA/B+, 105 SSA/B–) and 441 controls				Fixed effect		Random effects		Homogeneity
	SNP	MAF cases	MAF controls	OR	*P*	MAF cases	MAF controls	OR	*P*	OR	*P*	OR	*P*	*P*
A. All pSS cases versus healthy controls	rs11575837	0.017	0.041	0.41	0.0021	0.0080	0.010	0.80	0.66	0.48	0.0039	0.5	0.026	0.25
	rs2736191	0.025	0.058	0.46	0.0013	0.025	0.032	0.75	0.31	0.56	0.0019	0.57	0.019	0.2
	rs2187668*	0.24	0.12	2.46	1.73×10^{-11}	0.33	0.13	4.91	2.9×10^{-27}	3.36	1.7×10^{-34}	3.46	0.0003	0.0005
B. SSA/B+ cases versus healthy controls	rs11575837	0.018	0.041	0.42	0.012	0.0015	0.010	0.15	0.075	0.38	0.0033	0.38	0.0033	0.36
	rs2736191	0.025	0.058	0.47	0.0083	0.029	0.032	0.88	0.67	0.63	0.028	0.64	0.16	0.13
	rs2187668*	0.30	0.12	3.93	1.3×10^{-19}	0.38	0.13	7.60	1.6×10^{-34}	5.3	1.5×10^{-50}	5.45	2.8×10^{-7}	0.003
C. SSA/B+ cases versus SSA/B– cases	rs11575837	0.018	0.015	1.18	0.73	0.0015	0.024	0.060	0.011	0.73	0.48	0.31	0.43	0.01
	rs2736191	0.025	0.026	0.98	0.96	0.029	0.014	2.20	0.22	1.22	0.55	1.25	0.55	0.28
	rs2187668*	0.30	0.14	3.59	1.1×10^{-12}	0.38	0.18	4.56	1.3×10^{-10}	3.92	1.2×10^{-21}	3.92	1.2×10^{-21}	0.42

*rs2187668 is an SNP proxy for HLA-DRB1*0301 allele.

Table 2. Multi-SNP logistic regression adjusted for top two PCs, by cohort and combined via meta-analysis: (A) pSS cases versus healthy controls, (B) SSA/B+ pSS cases versus controls, and (C) SSA/B+ pSS cases versus SSA/B− pSS cases. SSA/B+ cases are SSA-positive or SSB-positive. SSA/B− cases are SSA-negative and SSB-negative.

		Exploratory (French) cohort		Replication (Scandinavian) cohort		Meta-analysis of two cohorts				
		574 pSS cases (340 SSA/B+, 233 SSA/B−) and 451 controls		436 pSS cases (329 SSA/B+, 105 SSA/B−) and 441 controls		Fixed effect		Random effects		Homogeneity
	SNP	OR	*P*	OR	*P*	OR	*P*	OR	*P*	*P*
A. All pSS cases versus healthy controls	rs11575837	0.38	0.001	1.22	0.7	0.5	0.008	0.63	0.4	0.06
	rs2736191	0.43	0.001	1.05	0.9	0.62	0.014	0.66	0.35	0.001
	rs2187668*	2.47	2.0×10^{-11}	4.93	8.2×10^{-27}	3.37	5.0×10^{-34}	3.48	0.0003	0.001
B. SSA/B+ cases versus healthy controls	rs11575837	0.37	0.007	0.29	0.27	0.36	0.0036	0.36	0.0036	0.8
	rs2736191	0.40	0.004	1.44	0.28	0.72	0.15	0.75	0.7	0.006
	rs2187668*	4.00	1.3×10^{-19}	7.67	1.1×10^{-33}	5.36	1.6×10^{-49}	5.52	1.6×10^{-7}	0.004
C. SSA/B+ cases versus SSA/B− cases	rs11575837	1.1	0.8	0.06	0.013	0.69	0.4	0.31	0.4	0.02
	rs2736191	1.17	0.7	3.12	0.09	1.52	0.2	1.66	0.28	0.2
	rs2187668*	3.56	1.6×10^{-12}	4.85	7.1×10^{-11}	3.98	1.4×10^{-21}	3.98	1.5×10^{-20}	0.3

*rs2187668 is an SNP proxy for HLA-DRB1*0301 allele.

We then combined results from the cohorts via two meta-analysis methods. Although an assumption of a single fixed effect across studies is appropriate in cases without evidence of heterogeneity, the alternative of estimating random effects across studies is not well powered given our small number of studies and low frequency of the *NKp30* SNPs. Thus, we present both models in Tables 1 and 2. These combined analyses indicated that both *NKp30* variants, rs11575837 and rs2736191, were inversely associated with risk of pSS, that is, they were protective, with OR = 0.48 ($P = 0.0039$) and OR = 0.56 ($P = 0.0019$), respectively, assuming fixed effects (Table 1). The association between rs11575837 and pSS was stronger and more statistically significant among the anti-SSA/B subset of patients (OR = 0.38, $P = 0.0033$) (Table 1).

To assess the independence of associations with the two NKp30 SNPs and the HLA-DR3 tag SNP, we also performed multi-SNP analyses modeling all three effects in a single logistic regression, again for both cohorts individually and then combined using meta-analysis methods (Table 2). NKp30 SNPs rs11575837 and rs2736191 are significantly associated in fixed-effects multiple-SNP models (thus adjusting for the HLA-DR3 tag SNP) with OR = 0.50 ($P = 0.008$) and OR = 0.62 ($P = 0.014$), respectively. However, there is significant heterogeneity and a lack of significant independence in the random-effects models. With cases restricted to anti-SSA/B–positive patients, there is homogeneity of association and a stronger and more significant effect (OR = 0.36, $P = 0.0036$), regardless of the method used.

Reduced transcription of NKp30 in individuals carrying the promoter variant

Because both protective SNPs were located within the promoter region of NKp30, we next assessed whether they could influence transcription levels of NKp30. NKp30 mRNA levels were investigated in 102 pSS patients from the French ASSESS cohort. Among them, 24 carried the minor allele rs2736191C, 12 carried the minor allele for rs11575837A, and 66 were homozygous for both major alleles (G and G, respectively). Patients carrying the minor allele for rs11575837 had lower expression levels of NKp30 mRNA compared to pSS patients carrying the major allele [median ratio NKp30/TBP (housekeeping gene): 1.432 and 1.801, respectively; $P = 0.04$; Fig. 1]. Thus, the minor A allele of rs11575837, which is protective (underrepresented among pSS patients), is associated with decreased transcription levels of NKp30.

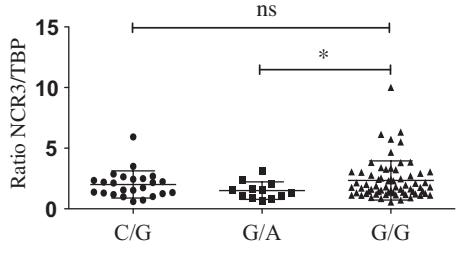

Fig. 1. The rs11575837 minor allele (A) is protective for pSS risk and is associated with reduced NKp30 expression levels. Relative NKp30 mRNA expression in peripheral blood mononuclear cells (PBMCs) from patients with pSS, according to genotype: C/G, carriers of rs2736191C minor allele and rs11575837G major allele; G/A, carriers of rs2736191G major allele and rs11575837A minor allele; G/G, carriers of both major alleles. Results represent means ± SEM. *$P < 0.05$. ns, nonsignificant.

Elevated NKp30 expression and function in pSS

We performed phenotypic characterization of NK cells in 38 pSS patients [92% women; mean age (±SD), 57 years (±15.3)] compared to 30 age-matched controls [43% women; mean age (±SD), 58 years (±16.4)]. Among pSS patients, the mean European League Against

Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI) was 3.1 (±4.1) (*28*), which is indicative of low to moderate activity. Twenty-two patients (57.9%) had a history of systemic involvement, including lymphoma in 2 patients (5%). pSS is affecting more specifically women (sex ratio male/female: 1:9); thus, we focused our phenotypic study on the data obtained from diseased (pSS) and control (HV) females.

Lymphopenia is a well-known abnormality in pSS and was also observed in our study [mean lymphocyte count, 1389 (±555) and 1928 (±467)/mm^3 in pSS patients and controls, respectively; $P = 0.0072$; Fig. 2A, left panel]. The degree of lymphopenia was associated with disease activity [mean lymphocyte count, 1098 (±495) and 1598 (±528)/mm^3 in ESSDAI ≥4 or <4, respectively; $P = 0.0124$; Fig. 2A, right panel].

The proportion of circulating CD3$^-$CD56$^+$ NK cells does not differ between pSS patients and controls (Fig. 2B). Similar to many other autoimmune disorders, the proportion of NK CD56^{bright} NK cells was increased among pSS patients compared to controls [mean % of 9.8 (±5.9) and 5.5 (±3.8), respectively; $P = 0.02$; Fig. 2C], though this was not associated with disease activity among cases. The expression pattern of most inhibitory or activating NK cell receptors was not significantly different in pSS cases compared with controls (fig. S2), except for the NKp30-activating receptor that was significantly increased in pSS [mean MFI NKp30: 62.2 (±22.6) and 42.7 (±24.5), pSS versus controls, respectively; $P = 0.018$; Fig. 2E]. Similar conclusions were drawn comparing all our pSS and controls (male and female). A second series of pSS patients with autoantibodies ($n = 18$) corroborated these data, showing that the MFI of NKp30 in circulating NK cells from pSS with positive anti-SSA was higher than that of matched controls ($n = 16$) (mean MFI, 446 versus 263, respectively; $P = 0.004$).

To assess the functional relevance of increased expression levels of NKp30 on NK cells, we performed a receptor cross-linking assay to analyze degranulation and IFN-γ secretion at various nonsaturating concentrations of agonistic anti-NKp30 antibody (and isotype or anti-CD16 antibody as controls). Eighteen individuals were assessed for correlation between the expression levels of NKp30 and CD107a, as a functional marker of degranulation, or IFN-γ release upon NKp30 cross-linking. At various concentrations of NKp30 antibodies, we observed a strong correlation between the proportion of NK cells expressing NKp30 with the percentage of NK cells secreting IFN-γ (Fig. 3), but not with the percentage of degranulating NK cells (fig. S3). No significant association was seen upon cross-linking with anti-CD16 antibody (Fig. 3, right panels).

Next, we addressed the functional relevance of harboring the minor allele of the rs11575837 (11575837A) for the NKp30-dependent IFN-γ release in four individuals. As expected, NK cells from these four carriers were hyporesponsive to NKp30 cross-linking despite normal to high expression levels of NKp30 (Fig. 3, red dots). Together, these data suggest that

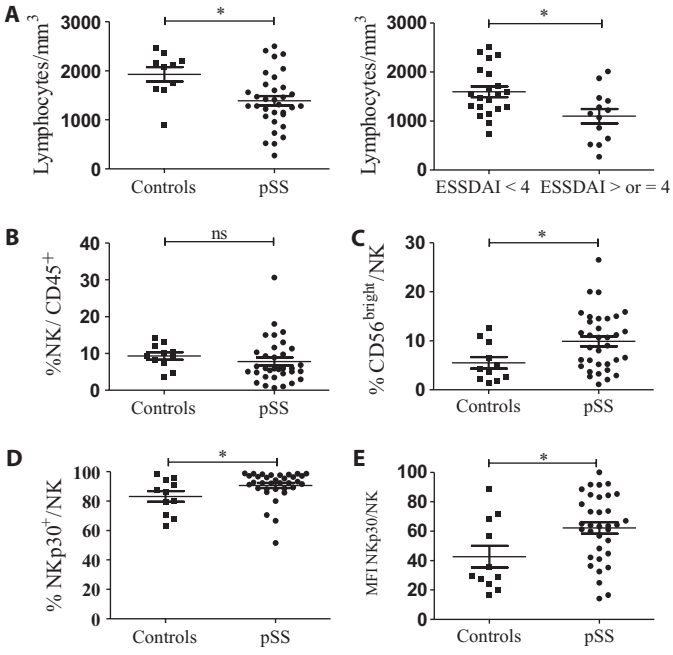

Fig. 2. Elevated expression of NKp30 among pSS patients. Phenotypic characterization using flow cytometry analyses of 34 diseased (pSS) and 11 control (HV) females. (**A**) pSS patients are lymphopenic (left panel), and this lymphopenia is more severe in patients with active disease defined by an ESSDAI ≥ 4 (right panel). (**B** and **C**) The proportion of circulating CD3$^-$CD56$^+$ NK cells (gated on CD45$^+$ cells) does not differ between pSS patients and controls (B), but the proportion of CD56^{bright} cells (gated on NK cells) is increased in patients (C). (**D** and **E**) The NKp30-activating receptor is significantly overexpressed in NK cells [in percentages (D) and in mean fluorescence intensity (E)] in pSS patients. Results represent means ± SEM. *$P < 0.05$. ns, nonsignificant.

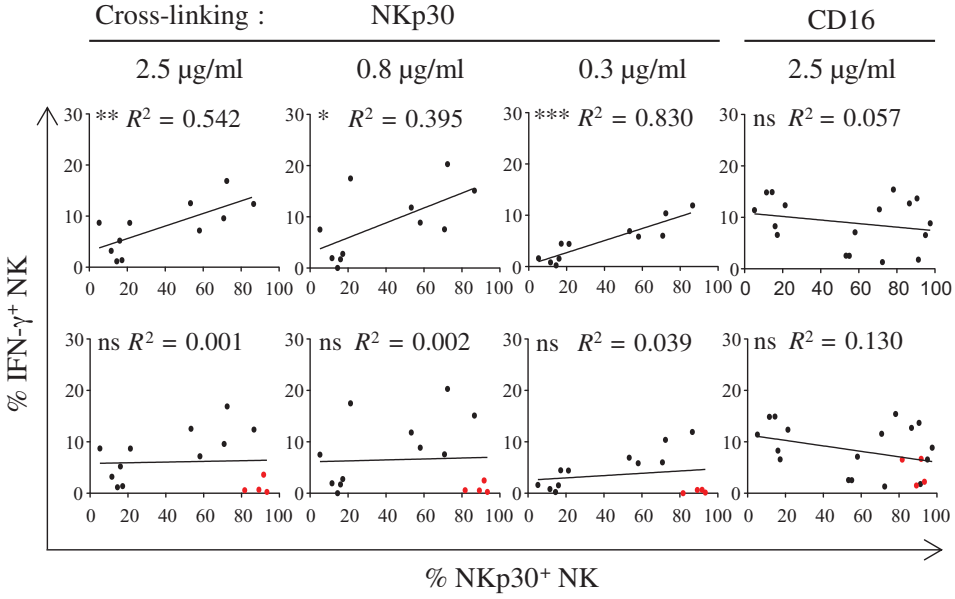

Fig. 3. Functional relevance of a high expression level of NKp30 on NK cells. Proportions of IFN-γ–positive NK cells determined using intracellular stainings in flow cytometry analyses after a 24-hour stimulation of PBMCs with NKp30 or FcγRIII (CD16) (right panels) antibodies. Linear regression with NKp30 expression levels. A dose response [using anti-NKp30 antibody (2.5, 0.8, and 0.3 µg/ml) coated onto plastic dishes] is depicted, each dot representing one patient or control. Four individuals carrying the minor allele of the rs11575837 (11575837A) were assessed in parallel, and the results are indicated in red dots. *$P < 0.05$, **$P < 0.001$.

circulating NK cells are prone to exert potent NKp30-mediated IFN-γ release upon engagement of the respective ligands. Individuals harboring the minor allele of rs11575837 exhibit reduced IFN-γ secretion upon NKp30 triggering.

Accumulation of NK cells in MSGs

A hallmark of pSS is the presence in MSGs of inflammatory foci. The focus score represents a standardized method for semiquantifying this process and involves counting the number of such foci (consisting of at least 50 mononuclear cells) per 4 mm^2 of glandular tissue (*29*). We analyzed the degree and topography of the NK cell infiltration in MSG biopsies (MSGBs) harvested from 7 controls (sicca syndrome with normal salivary gland biopsy or nonspecific sialadenitis and without autoantibodies) and 20 pSS patients. Staining of paraffin-embedded tissue sections using anti-NKp46 antibody revealed increased numbers of tissue-resident NK cells (*25*, *30*) ($P = 0.026$ comparing pSS and controls; Fig. 4, A to C). In pSS patients, these NK cells accumulated mainly outside of the inflammatory foci in the same areas as plasmacytoid DC-like cells (*5*). Moreover, we observed a correlation between the grading of focus score and the number of NKp46-positive cells outside of the inflammatory foci ($r^2 = 0.425$; $P < 0.0001$; Fig. 4, B and C).

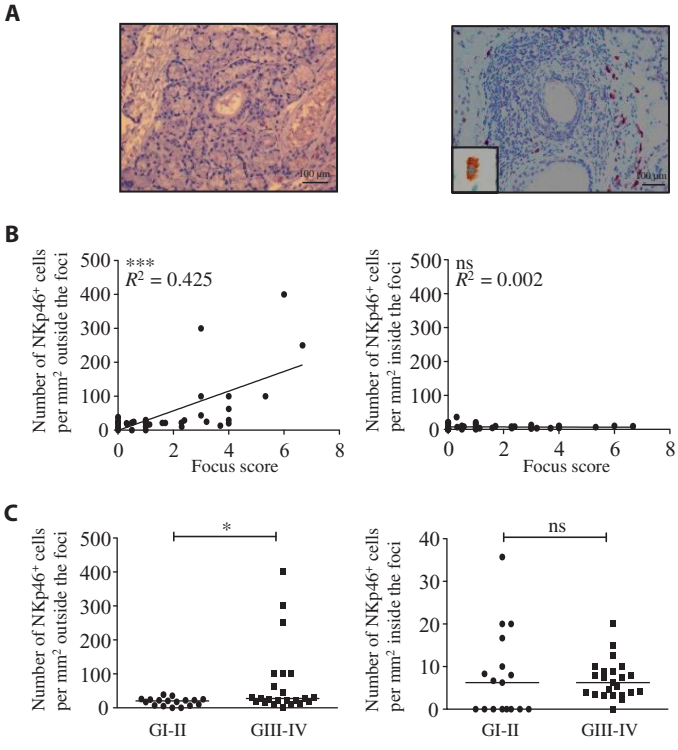

Fig. 4. Accumulation of NK cells in MSGB. (**A**) Representative NKp46 staining of a paraffin-embedded specimen from pSS and sicca patients. pSS (right micrograph pictures) and sicca controls (left panel): magnification, ×250 and ×945 (inset) (right panel) or ×200 (left panel). Scale bars indicated. (**B** and **C**) Correlation between NK cell numbers and focus score (B) or grade (C) in different areas of the glands. In the Chisholm-Mason scale (*54*), a grade III (equivalent to 1 focus/4 mm^2) or IV (equivalent to >1 focus/4 mm^2) is suggestive of the diagnosis of pSS. The numbers of NKp46-positive cells within (right panels) versus outside the foci (left panels) were evaluated in 40 fields observed at ×200 power of magnification in 23 MSGB grade III and IV and 17 MSGB grade I and II. Each dot represents one MSGB. *$P < 0.05$, Mann-Whitney *t* test.

NKp30-dependent cross talk between NK cells and epithelial cells within salivary glands

The levels of NKp30 expression were lower in NK cells residing in salivary glands of pSS compared with circulating NK cells (fig. S4A), suggesting a local NKp30 engagement. However, the transcription of the NKp30 gene remained unaltered (fig. S4B). Therefore, we assessed the expression of B7-H6, one of the NKp30 ligands, on salivary gland epithelial cells, the human salivary gland (HSG) cell line, and primary cultures of salivary glandular epithelial cells (SGECs) derived from MSGBs. B7-H6 mRNA was detectable in both pSS and sicca salivary glands at diagnosis (Fig. 5A). HSG cells were incubated with different proinflammatory cytokines known to play a role in pSS (*31*). TNF-α and IFN-γ were of special interest because they are secreted by NK cells upon engagement of NKp30. Incubation for 6 hours with TNF-α or poly(I:C) (polyinosinic-polycytidylic acid) (but not IL-17, IL-22, IL-23, or IFN-γ) significantly up-regulated B7-H6 mRNA levels on HSG and some cases of SGECs (Fig. 5B). Next, we assessed whether the interaction between NKp30 and B7-H6 was functional. HSG cells stimulated for 24 hours with TNF-α or poly(I:C) were incubated with Jurkat cells genetically modified to express one of the three major NKp30 isoforms or a control vector. The secretion of IL-2 was used as a marker of Jurkat cell activation. TNF-α promoted the cross talk between HSG and Jurkat-NKp30A (and to a lesser extent with NKp30B) in an NKp30 (but not NKp44 or DNAM)–dependent manner (Fig. 5D). We assessed the T_H1/T_H17 polarization of the effector cells contained in salivary glands of six pSS and sicca patients using qRT-PCR detecting cytokines (IFN-γ, IL-17, and IL-22) and transcription factors (Rorγt and T-bet). There was a trend toward a T_H1 pattern of gene expression in pSS patients (fig. S5).

DISCUSSION

Our findings implicate the activating NK cell–specific receptor NKp30 and its interaction with B7-H6 epithelial cells in the pathogenesis of pSS. Four lines of evidence support this assumption. First, a rare genetic variant residing in the promoter region is associated with reduced NKp30 mRNA expression levels and function upon NKp30 triggering and appears to protect against the development of pSS. Second, NK cells from pSS patients overexpress NKp30 receptor, which are associated with higher IFN-γ secretion levels upon engagement. Third, although NK cells are quite reduced in the blood, they are enriched in MSGs outside the inflammatory foci and correlate with the sialadenitis score. Last, B7-H6, the ligand for NKp30, is expressed by salivary epithelial cells and regulated by TNF-α or poly(I:C), triggering NKp30-mediated effector functions.

Classically, autoimmune diseases (AIDs) represent syndromes caused by an exaggerated activation of the adaptive arm of immunity (T and B cells), aimed at recognizing antigen-specific targets. Alternatively, AIDs could be conceived as abnormal states in which self and damaged tissues are abnormally released into the periphery, processed, and presented by DC to T cells that then migrate to the diseased organ. Thus, NK cells could play a crucial role in this process. Primary SS typifies this model. In inflamed salivary glands from pSS patients, apoptotic epithelial cells may release autoantigens such as Ro/La ribonucleoprotein complexes (*32*).

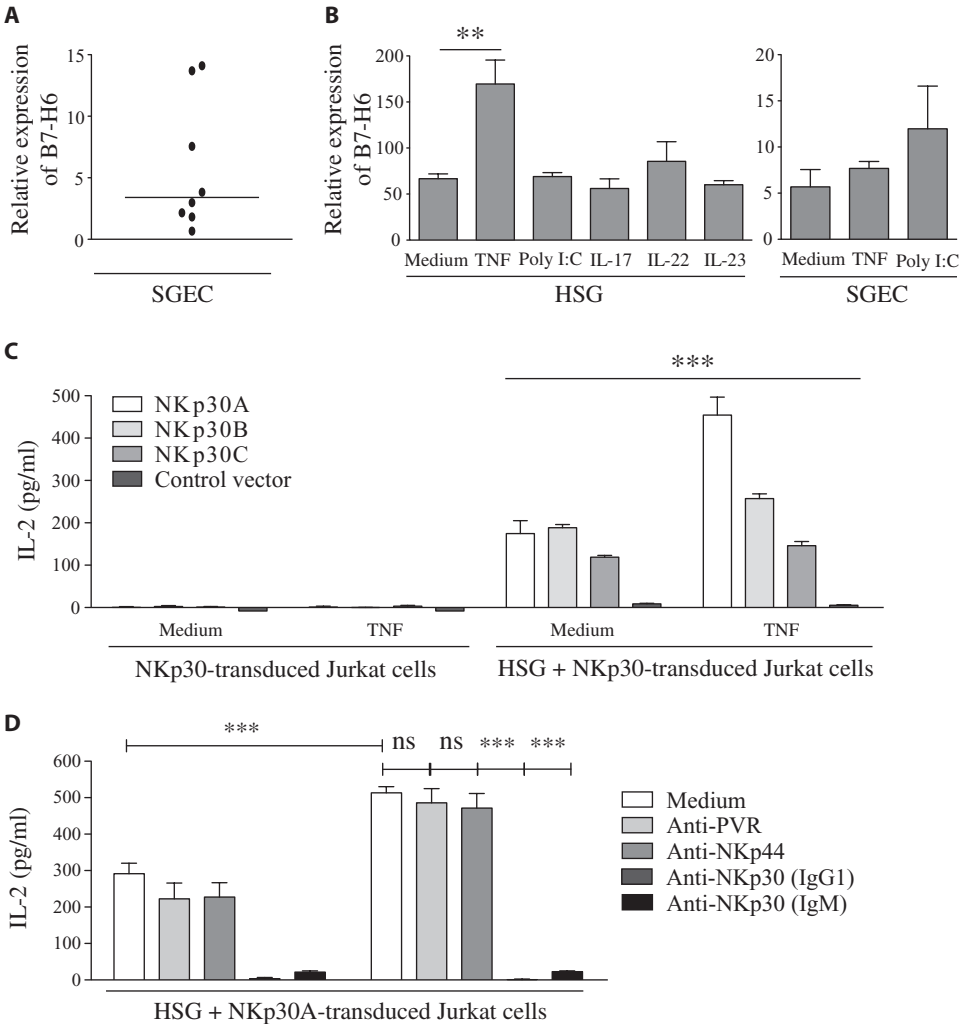

Fig. 5. TNF-α induces the cross talk between salivary glands and NK cells in an NKp30-dependent manner. (**A** and **B**) Transcriptional levels of B7-H6 in salivary glands and regulation. B7-H6 mRNA expression was assessed by qRT-PCR. B7-H6 mRNA expression was assessed from HSG cell line or SGECs derived from MSGB at baseline (biopsy of eight glands) (A) and after a 6-hour incubation with TNF-α (1 ng/ml), poly I:C (30 μg/ml), and IL-17, IL-22, or IL-23 (10 ng/ml) (B). (**C**) Functional cross talk between HSG- and NKp30-overexpressing Jurkat cells stimulated by TNF-α. TNF-α–stimulated HSG cells were incubated with Jurkat cells transduced with the three major NKp30 isoforms or with the control vector for 24 hours. The secretion of IL-2 was monitored by enzyme-linked immunosorbent assay (ELISA), and a histogram of two independent experiments is shown. (**D**) Role of NKp30 ligand/NKp30 interaction in the dialogue between HSG and transduced Jurkat cells. NKp30A-transduced Jurkat cells were incubated with anti-NKp30 or irrelevant blocking antibody for 2 hours before stimulation with HSG [as described in (B)]. A representative histogram of two experiments is shown. $^{**}P < 0.001$, $^{***}P < 0.0001$, two-way analysis of variance (ANOVA).

These autoantigens participate in the activation of B and T cells that lead to tissue damage. In this model, NK cells would be activated by ligation of their activating receptors recognizing stress-induced molecules in the damaged tissues, thereby activating immature DCs in the lymph node and eliciting the priming of effector T cells that can destroy peripheral target tissues. To date, research focusing on NK cell numbers, phenotypes, and functions in AID has been mostly correlative and characterized by a number of pitfalls and limitations. First, circulating NK cells rather than tissue-residing NK cells have been studied because of limited access to inflamed tissues. Second, some analyses of NK cells have failed to distinguish bona fide NKp46-positive NK cells from NKT cells and T cells expressing NK receptors. Third, antibodies and technical methods specifically recognizing NK cells in paraffin-embedded tissues have not been available until very recently (*25*, *30*). Fourth, there is still no perfect animal model of pSS, and those recently described (*33*–*35*) have not specifically studied the role of NK cells. Moreover, mouse and human NK cells may diverge at several levels (including NKp30, which is a pseudogene in mice). Finally, improved methods for pSS classification (*36*) and activity assessment (*28*) now allow for more consistency in disease characterization and status across studies. Despite these limitations, direct involvement of NK cells has been found in some human immunopathologies such as macrophage activation syndromes, antigen processing deficiencies, multiple sclerosis, and psoriatic arthritis (*37*–*43*).

Results of our case-control genetic study support a role for NK cells in pSS because genetic polymorphisms residing within the promoter region of NKp30 are associated with reduced risk of disease. The association between 11575837A and pSS was even stronger among patients whose disease is characterized by specific autoantibody production. This could result from the central role of NKp30 in DC maturation (*44*), thus promoting the priming process. The rs2736191 SNP was found to be associated with a more severe form of malaria (*45*). The GGTCCT sequence containing the rs2736191 polymorphic site is a RREB1/LZ321 binding motif. The polymorphism could cause the loss of this binding site, leading to decreased levels of transcription. In the context of acute malaria, this decrease in NKp30 expression is deleterious. Conversely, because of the possible deleterious role of NKp30-expressing NK cells in AID, such a variant could be protective in the context of chronic inflammation. Although there was no decrease of NKp30 mRNA in patients carrying the rs2736191C allele, this was the case among patients carrying the minor A allele of the rs11575837 SNP, which is associated with reduced IFN-γ secretion upon NKp30 triggering. Notably, the mechanisms linking the SNP of the promoter and the reduced IFN-γ secretion upon NKp30 triggering remain to be understood; one hypothesis is the presence of another polymorphism in linkage disequilibrium (LD) with the rs11575837. Thus, the presence of the major G allele, which was more frequent in pSS patients than in controls, could lead to increased levels of NKp30 mRNA expression favoring IFN-γ secretion upon triggering by ligands.

Our data support the fact that a functional interaction of B7-H6 or other NKp30 ligands with NKp30 receptors is relevant in salivary glands of pSS patients. First, B7-H6 is expressed by freshly isolated epithelial cells of pSS patients. The transcriptional activity of the B7-H6 gene can be up-regulated by poly(I:C) or TNF-α. Second, NKp30 is down-regulated in NK cells from the pSS glands (but at the transcriptional level, NKp30 transcripts remain detectable) compared with circulating NK cells. Third, pSS glands are markedly enriched in NK cells compared with non-pSS glands and located outside of the inflammatory foci, where DC-SIGN$^+$ DCs and plasmacytoid DCs have been described [(*5*) from our group, (*1*)]. Our results suggest a positive feedback loop between TNF-α– and IFN-γ–producing NK cells and B7-H6 expression on epithelial cells in the neighboring acini. This cross talk could be a key early event in the pathogenesis of the disease, leading to an increase of all the cytokines (IFN-γ, IL-17, and BAFF) who are going to secondarily promote migration of T and B cells.

The pathogenic role of immune infiltrates in pSS development has been addressed in mouse and human studies. Several reports in mouse models of autoimmune exocrinopathy mimicking human pSS supported the role of the T_H17/IL-23 axis in the exocrinopathy (*12*, *46*), whereas IL-27 ameliorated the syndrome (*47*). In human pSS, the cellular origin (T_H17, double-negative T cells) of IL-17 remains controversial (*10*, *13*). The recent work from Triolo's group elegantly showed using immunohistochemistry, qRT-PCR, and flow cytometry that NK cells are the major sources of IL-22 in MSGs from pSS patients (*11*). In our small series of six patients, the qRT-PCR profile of pSS glands indicated high levels of transcription of T_H1 cytokines (TNF-α, IFN-γ, and Tbx21) in pSS compared with sicca syndrome, whereas RORγt transcription was elevated but nondifferent between the two groups. Our flow cytometry analyses of leukocytic infiltrates of salivary glands failed to reveal T_H17 cells but identified CD3$^-$CD56$^-$CD45$^+$ cells as potential IL-22 producers (fig. S6). Last, should IL-17 and IL-22 be present in salivary glands, they do not play a major role in the cross talk between epithelial cells and NK. Indeed, in contrast to TNF or poly(I:C), most cytokines of the IL-17/IL-23 axis failed to up-regulate B7-H6 in the HSG salivary epithelial cell line. Such variabilities in the reported findings might stem from the precise kinetics of the NK and T cell homing and cytokine release in the inflamed tissues at the different stages of pathogeny of the disease [as exemplified in the mouse C57BL/6.NOD-Aec1Aec2 (*12*)].

Together, these results strongly implicate the NKp30/B7-H6 axis in the pathogenesis of pSS. Increased NKp30 signaling based on genetic predisposition could contribute to the IFN signature and the characteristic exocrinopathy. More work will be needed to elucidate the causal mechanism of NK cells in the induction phase of this AID and to determine whether the NKp30/B7-H6 interaction is drug targetable.

MATERIALS AND METHODS

Patients and controls

NCR3/NKp30 polymorphisms were studied among patients from the French ASSESS and Hôpitaux Universitaires Paris-Sud cohorts and French healthy blood donors (574 patients and 451 control subjects) (exploratory cohort) and among a Scandinavian cohort of pSS patients and controls of Caucasian ancestry (replication cohort). pSS patients fulfilled American-European Consensus Group criteria (*36*). The Scandinavian cohort includes a total of 436 Caucasian pSS patients from Sweden (n = 244) and Norway (n = 192) and 441 healthy controls (Sweden, n = 232; Norway, n = 209). The study was approved by the local research ethics committee, and informed written consent was obtained from all patients and controls.

We obtained blood samples from 38 pSS patients to perform phenotypic characterization, and 16 pSS patients to perform functional assays. Patients were referred to the Department of Rheumatology of Hôpitaux Universitaires Paris-Sud between October 2010 and April 2013. Patients treated with immunosuppressive drugs and/or corticosteroid therapy (≥10 mg/day) were excluded. For functional assays, exclusion criteria were more rigorous because drugs could impair NK cell function (*48*). Thus, patients treated with nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroid therapy at any dose were excluded. Disease activity was assessed with the ESSDAI (*28*), and a score ≥4 defined active disease. Patients referred to the department for mechanical back pain were used as controls, including 30 age-matched patients for phenotypic characterization and 10 patients for functional assays. Exclusion criteria for controls were history of autoimmune disorders or recent neoplasia (in the last 5 years), current biological inflammatory syndrome, and current corticosteroid therapy or NSAIDs.

Genotyping and quality control

After isolating genomic DNA from PBMCs using EZ1 DNA blood kits (Qiagen), the following SNPs were genotyped (table S1): nine SNPs encompassing the *NCR3 locus (6p23)* and three additional SNP proxies for HLA-DR2, HLA-DR3, and TNF, given the extensive LD within the major histocompatibility complex (MHC) region and known association of pSS with MHC region variants (*23*, *24*). Genotyping used a predesigned TaqMan assay from Applied Biosystems (assay no. 26882391-1) with a competitive allele-specific PCR system (KASpar genotyping, http://www.kbioscience.co.uk/).

Subjects with individual genotyping call rates <0.85 were removed as part of quality control (QC) measures. Duplicate samples/individuals were also removed. Principal components (PC) analysis based on 47 ancestry informative markers (AIMs) was used to detect population outliers in the French cohort. After using data from AIMs, applying QC measures and outlier removal, the exploratory cohort consisted of 574 pSS patients from the French ASSESS Cohort or followed at Bicêtre's teaching hospital and 451 independent control subjects.

The Scandinavian cohort was genotyped by single-base extension with fluorescence polarization template dye incorporation. The replication study used PC analysis with 3410 independent genetic markers from the Immunochip (r^2 < 0.2) to remove subjects with evidence of non-European ancestry, resulting in 436 Caucasian pSS patients and 441 healthy controls available for analysis. Both cohorts used EIGENSTRAT (*49*) for PC analysis and outlier detection, which was also used for ancestry adjustment in logistic regression analyses (see "Statistical analysis" section).

qRT-PCR and nonhierarchical clustering

Total cellular RNA was isolated from PBMCs with the RNeasy kit. First-strand complementary DNA (cDNA) was synthesized from 5 μg of total RNA with SuperScript III reverse transcriptase and random primers according to Invitrogen's instructions. The PCR primers and TaqMan probes for the six NKp30 transcripts and the β_2 microglobulin housekeeping transcript were designed with Primer Express software version 1.0 (Life Technologies) as previously described (*25*). For each NKp30 isoform, standard curves based on six data points of NK92 cDNA dilutions in triplicate were established to estimate the efficiency of PCR

amplification with StepOne software version 2.0. The efficiencies of NKp30A, NKp30B, and NKp30C PCR were 88.8, 90.6, and 93.8%, respectively. The qRT-PCR data were analyzed with the $2^{-\Delta\Delta Ct}$ method (*50*). Proportions of the distinct NKp30 isoforms were determined as the ratio of the relative quantities of each isoform and the total quantity of the three isoforms. Unsupervised hierarchical clustering was applied to data that had been log-transformed and median-centered with the Cluster and TreeView programs (average linkage clustering with Pearson's centered correlation as similarity metric) (*51*). The χ^2 test was used to assess association between isoform profiles and pSS phenotypes. Total NKp30 mRNA levels were determined with Bio-Rad Real-Time PCR systems. TBP (TATA box–binding protein) was used as a housekeeping gene. PCR primers and TaqMan probes were designed by Life Technologies. Primers for *NCR3* were specific to exons 1 and 2, which allowed for the amplification of all three NKp30 isoform transcripts, leading to the assessment of the global quantity of NKp30 transcripts irrespective of isoform profiles.

NK cell functional characterization

Phenotyping. Nine-color flow cytometry analysis was performed on whole blood cells and on PBMCs. The following mouse anti-human antibodies were used (table S3): from Miltenyi: anti-CD8–FITC (fluorescein isothiocyanate), anti-NKp30–PE (phycoerythrin), anti-CD4–PerCP (peridinin chlorophyll protein), anti-NKG2D–APC (allophycocyanin), anti-NKp44–PE, anti-NKp46–APC, anti–CD158b (KIR 2DL2)–PerCP, anti–CD158e/k (KIR 3DL1)–PE, anti–CD158i (KIR 2DS4)–APC, and anti–CD158a/h (KIR 2DL1)–FITC; from Beckman Coulter: anti-CD3–APC750, anti-CD56–PE-Cy7, anti-CD56–APC, anti-CD16–PB (Pacific Blue), and anti-CD117–PE-Cy7; from R&D Systems: anti–IL-23R–FITC, anti–DNAM-1–FITC, anti-NKp80–FITC, anti-LAG3–FITC, and anti-NKG2A–FITC; from BD: anti-CD45–PE-Cy7 and anti-CCR6–PerCP-Cy5.5. Dead cells stained by Vivid Yellow (Molecular Probes, Invitrogen) were excluded before analysis. The following isotype controls were used: mouse immunoglobulin G1 (IgG1) FITC, PE, and APC. Before the staining, PBMCs were FcR-blocked (Miltenyi) for 30 min. Stained cells were acquired within 24 hours on a Cyan Flow Cytometer (Beckman Coulter), and analyses were performed with FlowJo software (Tree Star). Results were expressed as percentage of cells positive for each marker and/or MFI to assess the density of receptors expressed on the cell surface.

IFN-γ production and CD107a degranulation assays. NK cells were stimulated by NKp30 cross-linking without purifying NK cells from PBMCs as previously described (*52*). Briefly, PBMCs from patients and controls were prepared according to standard procedures on Ficoll-Hypaque. Monoclonal antibodies anti-NKp30 agonist and mouse IgG2a isotype control (R&D Systems) were coated onto flat-bottomed 96-well Maxisorb plates (Nunc) overnight at 4°C. Three coating concentrations of anti-NKp30 were used: 2.5, 0.8, and 0.3 μg/ml. The specificity of the responses observed in our assay was tested with isotype control reagents as background controls and an additional cross-linking assay with anti-CD16 agonist (R&D Systems). Isotype control and anti-CD16 were coated at the maximal coating concentration (2.5 μg/ml). To assess CD107a degranulation, 2×10^5 PBMCs per well in RPMI 1640 and 10% fetal calf serum (FCS) were added onto anti-NK cell receptor antibody–coated plates in the presence of low-dose IL-2 (10 IU/ml). Triplicate wells were set up for each test condition. After 1 hour of incubation with anti-CD107a–FITC, the protein transport inhibitor GolgiStop (BD Biosciences) was added at a final dilution of 0.6 μl/ml. Cells were then incubated for an additional 4 hours at 37°C. Cells were then recovered, washed, and resuspended with phosphate-buffered saline into 5 ml of polystyrene round-bottomed fluorescence-activated cell sorting (FACS) tubes and stained for flow cytometry analysis (anti-CD45–APC-Cy7, anti-CD56–PE-Cy7, anti-CD3–PB, and viability marker Vivid Yellow). To assess IFN-γ secretion, PBMCs were preactivated in the presence of IL-2 (100 IU/ml) overnight. The next day, 2×10^5 cells per well of preactivated PBMCs were seeded in triplicate onto Maxisorb plates coated with anti-NK cell receptor antibody. After a total of 5 hours, NK cells were recovered and stained for cell surface phenotypic analysis as described earlier. For intracellular cytokine staining, cells were fixed, permeabilized, and labeled with anti–IFN-γ PerCP following the manufacturer's protocol (BD Biosciences). Stained cells were acquired within 24 hours on Cyan Flow Cytometer (Beckman Coulter), and analyses were performed with FlowJo software (Tree Star).

Immunohistochemistry staining of MSGBs

The most widely accepted grading system of MSGBs, termed focus score, records the number of foci of lymphoid tissue, defined as collections of 50 or more lymphocytes per 4 mm^2 (*29*, *53*). In the Chisholm-Mason scale (*54*), a grade III (equivalent to 1 focus/4 mm^2) or IV (equivalent to >1 focus/4 mm^2) is suggestive of the diagnosis of pSS. MSGBs from 20 pSS patients and 7 controls were graded according to the focus score and Chisholm-Mason scale and stained with anti-NKp46 to assess NK cell infiltration. Sections (3-μm thick) of formalin-fixed, paraffin-embedded MSGB were mounted on poly-L-lysine–coated slides, deparaffinized, and hydrated through graded alcohols to water. Sections were pretreated with tris-EDTA buffer (10 mM tris, 1 mM EDTA, pH 9) for 30 min in a 98°C water bath. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxidase (DAKO) for 10 min. The primary antibody, mouse IgG2b anti- human NKp46 monoclonal antibody (clone 195314, 5 μg/ml, R&D Systems), was incubated for 1 hour, followed by the secondary antibody Biotin $F(ab')_2$ Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch) for 30 min, and detected with 3-amino-9-ethylcarbazole substrate (Vector Laboratories), and the sections were counterstained with Harris's hematoxylin and the slides were mounted with Glycergel (DAKO). Negative controls were made by substituting primary antibody with isotype controls.

Cell lines

HSG is a cell line derived from neoplastic epithelial duct cells of the human salivary gland [a gift of B. Baum and M. Kok (U.S. National Institutes of Health)], grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Jurkat cells, transduced with adenovirus containing the sequence for NKp30 A, B, and C isoforms or with vector control (provided by D. Klatzman), were cultured in RPMI 1640 plus 10% FCS, 1% sodium pyruvate (Gibco), and 1% penicillin/streptomycin.

Cultures of SGECs

Primary cultures of SGECs were established from MSGs from eight patients (three pSS and five sicca) as described (*55*). In brief, each lobule was cut into small fragments and set in six 75-cm^2 flasks with basal epithelial medium (a 3:1 mixture of Ham's F-12 and DMEM) supplemented with 2.5% FCS, epidermal growth factor (10 ng/ml), hydrocortisone (0.4 μg/ml), insulin (0.5 μg/ml), penicillin (100 IU/ml), and streptomycin (100 μg/ml) and incubated at 37°C under 5% CO_2. After 4 to 5 weeks of culture, at 70 to 80% confluence, cells were tested for the expression of B7H6.

Assessment of NKp30/B7H6 interaction within salivary glands

Coculture between HSG and Jurkat cells transduced with NKp30 isoforms. An HSG cell line was seeded overnight at 5×10^4 cells per well in 96-well flat-bottom culture plate in DMEM/F-12 medium supplemented with 10% FCS (PAA) and 1% penicillin/streptomycin antibiotic (Gibco). HSG and transduced Jurkat T cells were cocultured at a ratio of 1:1 for 24 hours in complete RPMI 1640 at 37°C. In some experiments, the NKp30-transduced Jurkat T cells were preincubated with anti-NKp30 (IgG1 or IgM), anti-NKp44 (IgM), or anti-PVR [IgG1, all used at 10% (v/v), provided by C. Bottino] for 2 hours at 37°C before the coculture. Supernatants were harvested to measure IL-2 cytokine levels with commercial ELISA (BD OpTIEA, CliniSciences).

Measurement of B7-H6 mRNA levels by qRT-PCR. First-strand cDNA was synthesized as described earlier. The PCR primers and TaqMan probes for the B7-H6, Bat3, and PPIA housekeeping transcripts (TaqMan Probe) were designed with Primer Express software version 1.0 (Life Technologies) or by Life Technologies (table S4). The qRT-PCRs were performed on StepOne following the manufacturer's recommendations (Life Technologies) and were analyzed with the $2^{-\Delta\Delta Ct}$ method (*50*).

Statistical analysis

Results are shown as means ± SD. The Mann-Whitney test was used to compare independent samples. The Wilcoxon signed-rank test was used to compare two related samples or for repeated measurements of a single sample. Linear regression was performed. Statistical comparisons were performed with StatView version 5.0 (SAS Institute Inc.). A P value <0.05 was considered significant. Analysis of single-SNP associations was performed in PLINK, and multivariate associations were performed in Stata 11. First, PCs reflecting ancestry were generated (after outlier removal) with EIGENSTRAT (*49*), from 47 AIMs in the exploratory cohort and 3410 independent SNPs in the replication cohort. Each cohort (French and Scandinavian) was analyzed separately via logistic regression, adjusting for the top two ancestry PCs. Results were then combined with fixed- and random-effects meta-analysis in the Stata metan function, which also produced the χ^2 test for homogeneity. SNPs were first analyzed individually in single-SNP analyses (one regression per SNP) and then together in multi-SNP analyses (one regression with all three SNPs) to investigate independence of effects.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/5/195/195ra96/DC1
Fig. S1. Expression of NKp30 isoforms in a cohort of pSS patients.
Fig. S2. Phenotypic characterization of pSS patients.
Fig. S3. Functional relevance of a high expression level of NKp30 on NK cells and hyporesponsiveness of the rs11575837 minor allele.
Fig. S4. Down-regulation of NKp30 expression in NK cells residing in salivary glands.
Fig. S5. Salivary gland cytokine/transcription factor analysis by qRT-PCR indicates high levels of T_H1 cytokines in pSS patients.
Fig. S6. Intracellular IL-17/IL-22 secretion assay in salivary gland lymphocytes.
Table S1. SNPs subjected to genotyping.
Table S2. Exploratory cohort. Logistic regression results adjusted for PC1 and PC2.
Table S3. Antibodies for flow cytometry analysis.
Table S4. PCR primers and TaqMan probes for the B7-H6 transcript.

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