

Humanized Mice as Models to study Human Innate Immunity and Immunotherapies

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ÉCOLE DOCTORALE Structure et dynamique des systèmes vivants (SDSV)





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ECOLE DOCTORALE n°577 : Structure et dynamique des systèmes vivants (SDSV)

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HUMANIZED MICE AS MODELS TO STUDY HUMAN INNATE IMMUNITY AND IMMUNOTHERAPIES

Paris, le 17 février 2017

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ABSTRACT/RÉSUMÉ

Animal models have extensively contributed to our understanding of human immunobiology and to uncover the underlying pathological mechanisms occurring in the development of the disease. However, mouse models do not always reproduce the genetic complexity inherent in human disease conditions. Human immune system (HIS) mouse models that are susceptible to human pathogens and can recapitulate human hematopoiesis provide one means to bridge the interspecies gap.

Severely immunodeficient host mice support life-long, high level human hematolymphoid development after engraftment with human hematopoietic stem cells (HSC). However, the differentiation and function of some blood cell types, including innate lymphoid cells (ILCs), is poorly characterized in current HIS mice. Here we describe the development of а novel HIS mouse model, named BRGSF (BALB/c $Rag2^{tm1Fwa}Il2rg^{tm1Cgn}Flk2^{tm1lrl}Sirpa^{NOD}$), which demonstrate enhanced maturation, function and homeostasis of human natural killer (NK) cells and other ILCs.

Furthermore, the BRGSF-based HIS mouse model recapitulated the developmental stages of human ILCs. We could identify for the first time an ILC precursor (ILCP) population that is present both in HIS mice and in human peripheral blood as well as in several lymphoid and non-lymphoid human tissues. This circulating human ILCP population may provide a substrate to generate mature ILCs in tissues in response to environmental stressors, inflammation and infection.

In a second part of the thesis we used BRGS (BALB/cRag2^{tm1Fwa}Il2rg^{tm1Cgnl}Sirpa^{NOD}) immunodeficient mice to assess two innate lymphocyte-based immunotherapeutic approaches for treating EGFR-expressing KRAS-mutated colorectal carcinoma in vivo. The first model used a combination of umbilical cord blood (UCB)-derived NK cells and the monoclonal antibody cetuximab to promote antibody dependent cell cytotoxicity (ADCC) against the tumors. In a second model, we evaluated the therapeutic suitability of novel bispecific VHH constructs that combine inhibition of the EGFR with the target-specific activation of effector V γ 9V δ 2-T cells.

These studies highlight the utility for HIS-based mouse models to understand human innate lymphocyte development and to harness these potent effectors for anti-tumor therapies. Les modèles animaux ont largement contribué à notre compréhension de l'immunologie humaine et des mécanismes pathologiques associés au développement des maladies. Cependant, les modèles murins ne permettent pas de reproduire toute la complexité des pathologies humaines. Les souris à système immunitaire humain (HIS), par leur capacité à récapituler l'hématopoïèse humaine et à être infectées par des pathogènes humains, constituent une solution de choix pour combler ce fossé inter-espèce.

Après greffe de cellules souches hématopoïétiques humaines, des souris hôtes sévèrement immunodéprimées permettent un haut niveau de développement du système hémato-lymphoïde humain tout au long de leur vie. Cependant, certains types cellulaires, comme les cellules lymphoïdes innées, ne parviennent pas à se différencier et à fonctionner normalement dans les modèles murins HIS actuels. Ici, nous décrivons le développement d'un modèle souris HIS original, nommé BRGSF (BALB/c *Rag2*^{tm1Fwa}*Il2rg*^{tm1Cgn}*Flk2*^{tm1lrl} *Sirpa*^{NOD}), montrant une amélioration de la maturation, de la fonction et de l'homéostasie des cellules natural killer (NK) humaines et des autres ILCS.

De plus, en récapitulant les différentes étapes du développement des ILCs humaines, ce modèle souris BRGSF nous a permis d'identifier pour la première fois un précurseur d'ILC (ILCP) présent à la fois dans notre modèle HIS ainsi que dans le sang périphérique et plusieurs organes lymphoïdes et non-lymphoïdes humains. Cette population circulante d'ILCPs pourrait constituer un substrat pour la production d'ILCs matures dans les tissus périphériques en réponse à des stress environnementaux, inflammatoires et/ou infectieux.

Dans une seconde partie de ce travail de thèse, nous avons utilisé ces souris BRGS afin de tester l'efficacité de deux immunothérapies reposant sur les lymphocytes innés pour le traitement d'un carcinome colorectal exprimant EGFR et muté pour KRAS. La première approche a consisté en la co-administration des cellules NK dérivées de sang de cordon ombilical et d'anticorps monoclonal cetuximab afin de promouvoir le mécanisme de cytotoxicité cellulaire dépendante des anticorps (ADCC) contre la tumeur. La seconde stratégie a reposé sur l'injection de nanobodies VHH combinant l'inhibition de l'EGFR et l'activation spécifique du récepteur V γ 9V δ 2 des cellules T effectrices.

Les résultats de cette étude soulignent l'importance des modèles murins HIS pour la compréhension du développement des lymphocytes innés humains et pour mieux les mettre à profit dans les thérapies anti-tumeurs

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LIST OF ABBREVIATIONS

ADCC	Antibody dependen cellular cytotoxicity
Ag	Antigen
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMP	Antimicrobial peptide
APC	Antigen presenting cell
BAC	Bacterial artificial chromosome
BLT	Bone marrow-Liver-Thymus
BM	Bone Marrow
BRG	BALB/c $Rag2^{tm1Fwa}Il2rg^{tm1Cgn}$
BRGS	BALB/c Rag2 ^{tm1Fwa} Il2rg ^{tm1Cgn} Sirpa ^{NOD}
CAR	Chimeric antigen receptors
CCL	(C-C motif)-chemokine ligand
CCR	(C-C motif)-chemokine receptor
cDC	Classical/conventional dendritic cell
CDP	Dendritic cell progenitor
CLP	Common lymphoid progenior
CLR	C-type lectin receptor
CMP	Common myeloid progenior
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
CRTH2	Prostaglandin D2 receptor 2
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	(C-X-C motif)-chemokine ligand
CXCR	(C-X-C motif)-chemokine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DETC	Dendritic epidermal T cells
DNAM	DNAX Accessory Molecule-1
EBV	Epstein–Barr virus
ESC	Embryonic stem cell
FL	Fetal liver
Flt3	Fms-like tyrosine kinase 3 ligand
FoxP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft-versus-host disease
HA	Hemagglutinin
HER	Human epidermal growth factor receptor 2
HIS	Human immune system

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hpre-cDC	Human convential dendritic cell precursor
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
ICOSL	Inducible T-cell costimulator
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
infDC	Inflammatory dendritic cell
IPP	Isopentyl pyrophosphate
iPS	Induced pluripotent stem cell
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer-cell immunoglobulin-like receptors
LFA	Lymphocyte function-associated antigen
LMPP	Lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharide
LTβR	Lymphotoxin-beta receptor
LTi	Lymphoid tissue inducer
MAIT	Mucosal associated invariant T
MAMP	Microbial-associated patterns
MAPK	Mitogen-activated protein kinase
MDP	Monocyte-DC progenitor
MDSC	Myeloid-derived suppressor cells
MEP	Megakaryocyte-erythrocyte progenitor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MISTRG	$C; 12984-Rag2^{tm1.1Flv}Csf1^{tm1(CSF1)Flv}Csf2/Il3^{tm1.1(CSF2,IL3)Flv}Thpo^{tm1.1(TPO)Flv}Il2rg^{tm1.1Flv}(SIRPA)^{Tg1Flv/J}$
MITRG	$C; 12984\text{-}Rag2^{tm1.1Flv}Csf1^{tm1(CSF1)Flv}Csf2/Il3^{tm1.1(CSF2,IL3)Flv}Thpo^{tm1.1(TPO)Flv}Il2rg^{tm1.1Flv}$
MLR	Mixed lymphocyte reactions
moDC	Monocyte derived dendritic cell
MPP	Multipotent progenitors
NCR	Natural cytotoxicity receptor
NF-ϰβ	Nuclear factor kappa-light-chain-enhancer
NKG2	Natural killer Group 2
NKP	Natural killer precursor
NKR	Natural killer receptor
NKT	Natural killer T
NLR	NOD-like receptor

NOD	Non-obese diabetic
NOG	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Sug} /Jic
NSG	NOD/.Cg-Prkdc ^{scid} IL2r ^{tm1wj} /Sz.j
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PDX	Patient derived xenograft
PGE2	Prostaglandin E2
PILR	Paired immunoglobulin-like type 2
PRR	Pattern recognition receptor
PSC	Pluripotent stem cell
RAG	Recombination activating gene
RANTES	Regulated on activation, normal T cell expressed and secreted
RLR	RIG-1 like receptors
RORg	RAR-related orphan receptor gamma
S1PR1	Sphingosine-1-phosphate receptor 1
SCID	Severe combined immunodeficiency
SIRPα	Signal regulatory protein alpha
SLO	Secondary lymphoid organ
SRR	SLAM-related receptor
STAT	Signal transducer and activator of transcription
TCF	T-cell factor
TCR	T-cell receptor
TGFβ	Transforming growth factor beta
Th	T helper
TL1A	TNF-like protein 1A
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
XCL	Chemokine (C motif) ligand
XCR	Chemokine (C motif) receptor

INTRODUCTION

Biomedical research has benefited from mouse experimentation to better understand mammalian biology since the 16th century, and it was after the generation of the first inbred mouse strains in the early 20th century that they were established as model organisms giving birth to many of the biological discoveries of the last 120 years. In many cases, this led to the development of successful treatments for previously untreatable diseases, for example acute promyelocytic leukemia¹. Although other model organisms are genetically closer to humans (such as dogs, pigs or non-human primates), mice are easier to maintain and breed, have short generation times and thanks to robust gene-modification approaches, provide an endless variety of mutants in several inbred backgrounds.

We know today that Jacques Monod and Francois Jacob's aphorism "Anything found to be true of E. Coli must be true of elephants" turns out to be too reductionist. Moreover, *M. musculus* and *H. sapiens* have been evolving divergently for 85 million years, adapting to very different environments thus undergoing selection for many traits, from the circadian rhythm to our body size ². Thanks to the decoding of mouse and human genomes in the early 2000, we now appreciate this independent evolution led to a difference in one-fifth of our genes. Not surprisingly, this part of the genome contains genes that suffered the highest selection pressure due to environmental contact such as human-microbe interactions. Indeed, the divergent sequences are involved in transcription regulation and chromatin organization with enrichment in the regions related to the immune system, metabolic processes and stress response ³.

It is therefore not unexpected that only 8% of the cancer studies in animal models reach clinical trials and that more than 80% of these eventually fail when tested in humans ⁴. The increasing knowledge of the molecular differences between mice and humans should allow us to evaluate the degree in which animal models may be suitable for translational research and when this is not the case, to then search for better systems.

Several reviews have carefully examined the differences between the immune systems of mice and humans ^{5,6} and highlighted the major importance of immune homeostasis for mounting an efficient immune response. To give some examples, the expression and sensitivity to TLR mediated challenge, the difference in the interferon-gamma (IFN- γ) signaling cascade, the composition of granule constituents in neutrophils and the different phenotype and molecules expressed by macrophages ⁷ differ in fundamental ways between mice and men, suggesting that the predictive value of mice disease models might be limited.

Should we abandon mouse research as a discovery tool for understanding human disease? Or can we adapt the mouse model to make it more relevant for human immunobiology? Chimeric xenografted mice provide an interesting opportunity to study some aspects of human immunity in a small animal model. Mice can be modified by introducing human genes or genomic regions and/or by transferring human cells. Throughout this thesis, I will use the term "humanized mice" for immunodeficient hosts that have been engrafted with human blood cells or blood-forming cells and organs that can partially recapitulate human hemato-lymphoid system development and function. These so called 'HIS' (Human Immune System) mice are an emerging tool to decipher human immune responses and novel immunotherapies.

I. THE HUMAN IMMUNE SYSTEM

EARLY HEMATOPOIESIS

I could use today the same words Alexander Maximow used in 1909 to define hematopoiesis: "an organized cellular hierarchy derived from a common precursor, the hematopoietic stem cell (HSC)". HSCs are responsible for the trillions of blood cells formed each day in adults. On the one hand, HSCs have the capacity to give rise to daughter HSCs without differentiation; on the other hand, HSCs can enter into a dynamic and wellorchestrated differentiation process that generates other progenitors⁸. This involves a gradual loss of differentiation potential modulated by the expression of transcription factors that ultimately guides the specialization of diverse hematopoietic lineages ⁹. Different models have been proposed defining specification branch points and timing of the generation of downstream progenitors (Figure 1). While the classical model splits differentiation into myeloid and lymphoid branches ^{10,11}, an alternative model proposes a three axes panorama derived from a common myeloid progenitor (CMP) and a lymphoid-primed multipotent progenitor (LMPP) (Figure 1)¹². The LMPP gives rise to the common lymphoid progenitor (CLP) that differentiates in T-, B-lymphocytes and innate lymphoid cells while the megakaryocyte-erythrocyte progenitor (MEP), derived from the CMP, produce platelets and erythrocytes. The novelty of this revisited model of hematopoiesis is that both the myeloid primed (CMP) and the lymphoid primed (LMPP) progenitors maintain the capacity to switch to the granulocyte-macrophage progenitor (GMP) that generates monocytes and macrophages, granulocytes and dendritic cells (DC).



Figure 1: Models of human hematopoiesis hierarchy. A) Classical model of the hematopoietic hierarchy with a strict separation between the myeloid and lymphoid branches as the first step in lineage commitment downstream of the hematopoietic stem cell. (B) Alternative model as proposed by Adolfsson et al, incorporating the identification of LMPPs.

ADAPTIVE IMMUNITY

The adaptive immune system is formed by two broad sets of antigen-responsive cells: B (bursal or bone marrow-derived) lymphocytes and T (thymus-derived) lymphocytes, which in the blood and secondary lymphoid organs can be identified as CD19⁺ and CD3⁺ cells, respectively. In steady state, naïve B and T cells harbor a highly diverse repertoire of antigen (Ag) specificities. Upon encounter with cognate antigen, they proliferate massively generating antigen-specific 'clones' that differentiate into the effectors of immunity. Upon antigen clearance, a fraction of 'memory' cells remain that is the basis for the antigenspecific recall responses, which has been classically considered a hallmark of adaptive immunity ^{14,15}. In the case of B cells the antigen specificities come from immunoglobulin receptors (Ig) that after engaging with an antigen, they trigger cell activation, clonal expansion and differentiation into antibody producing plasma cells. Antibodies are capable of recognizing three-dimensional structures and interact with pathogens leading to its neutralization. In contrast, recognition of antigen by T cells involves T cell receptor complex (TCR) engagement of peptides presented by class I and II major histocompatibility complex (MHC) molecules. These peptide/MHC complexes are expressed on the surface of antigen presenting cells (APCs), especially dendritic cells (DCs)¹⁶. Accordingly, DC are the 'gatekeepers' of immunity and they dictate immune response initiation and coordinate innate and adaptive immune activation.

Thymic selection generates two types of T cells: those expressing a TCR receptor formed by $\alpha\beta$ chains and those carrying $\gamma\delta$ -TCR. $\gamma\delta$ -T cells are much less understood and they are peculiar in that they have characteristics that place them in the border between innate and adaptive immunity. Their repertoire heterogeneity is poorer and they localize in very precise sites such as skin and some mucosal surfaces. The biology of $\gamma\delta$ -T cells will be further developed below.

On the basis of the co-receptor expressed $\alpha\beta$ -T cells are divided into CD8⁺ cytotoxic T cells and CD4⁺ helper T cells. Ag-activated helper T cells will differentiate into different phenotypes, depending on the stimulus and cytokines in the local environment, which can be characterized by their cytokine profile and by transcription factors. Th1 cells produce IFN- γ and interleukin-2 (IL-2) and express T-bet. Th2 cells produce IL-4, IL-5 and IL-13 and express GATA-3. Th17 cells produce IL-17 and IL-22 and express ROR γ t. A fifth type of "conventional" T cells, responsible for the maintenance of peripheral tolerance and down-modulation of immune responses, are called regulatory T cells (T_{reg}). These can be divided

into different subsets based on the expression of FoxP3 and/or the production of IL-10, TGF- β and IL-35. Recently a new equilibrium model of immunity has proposed where T_{regs} can differentiate into specialized type 1, 2 or 3 subsets depending on the associate effector axis triggering the action ^{17–20}.

INNATE IMMUNITY

Unlike the adaptive immunity that appeared later in evolution, the innate arm of the immune system developed already in the first multicellular organisms. Intimately related to their phylogeny is the mechanism these two systems use to recognize their target. By contrast to the specificity shown earlier for T and B-lymphocytes, innate effectors have been classically seen as "broad responders". Innate effector cells bear 'sensors' that are capable of recognizing conserved structures shared by large groups of pathogens (termed microbialassociated patterns (MAMPs) ^{21,22}such as the lipopolysaccharide (LPS) that is present in all gram-negative bacteria). As such, the innate sensors are referred to as pattern recognition receptors (PRRs). The four PRR families include Toll-like receptors (TLR), nucleotide oligomerisation receptors (NLR), C-type lectin receptors (CLR) and RIG-1 like receptors (RLR). In humans, TLRs include 10 members, each of them with a broad range of specificities (Figure 2)²³. Engagement with TLR ligands triggers a signaling cascade that concludes with the activation either of the MAP kinase, the NFkB or the IRF pathway. Eventually TLR stimulation leads to the production of pro- or anti-inflammatory cytokines, type I IFNs, chemokines and chemokine receptors, anti-microbial and co-stimulatory molecules and the enhance of the antigen uptake and presentation (Table 1)²⁴. Also noninfectious endogenous biomolecules called damage-associated molecular patterns (DAMPs) or alarmins can bind to TLRs promoting or exacerbating the inflammatory response in a context of stress. The fundamental role of TLRs has been evidenced by several studies that compared the disease susceptibility of individuals carrying different polymorphisms in genes that participate in TLR signaling (Figure 2). These included sepsis, immunodeficiencies, atherosclerosis and asthma, among others, suggesting a great therapeutic potential in the manipulation of these receptors ²⁵. Indeed, recent investigations have exploited TLRs as adjuvants in vaccines and as tumor immunotherapeutics and have also assessed the potential of TLR agonists in autoimmune and inflammatory diseases ^{26,27}. Like TLRs, CLRs are found in contact with the extracellular space surveying for the presence of microbial ligands. This greatly heterogeneous family shares the characteristic C-type lectin-like domain and its two best characterized members are Dectin-1 and Dectin-2. These receptors signal through ITAM-like domains in myeloid cells including DCs, macrophages, neutrophils and monocytes. They are fundamental in the recognition of fungal β -glucans and α -mannans, such as in *Candida albicans*, as well as patterns of Listeira and Mycobacterium²⁸. Contrarily

to the previous PRR families, NLRs and RLRs are located in the cytoplasm where they patrol for the presence of intracellular pathogens. NOD1 and NOD2 are the prototypical members of the NLR family and they both detect components of the bacterial outer membrane or cell walls^{29,30}. After binding to those ligands, a signaling complex is assembled leading to the activation of NF- $\varkappa\beta$ and MAPK pathways. The importance of this family of receptors is evidenced in Crohn's disease patients that express a NOD2 variant with impaired responsiveness and incorporation of bacteria within the autophagosomes. Finally, RLRs are a group of three helicases that detect the presence of foreign RNA within the cytosol. They do so by recognizing features common to viral genomes and replication intermediates, such as the poly-U region in the HCV^{31,32}. Their role is critical in the immune defense against RNA virus but they also participate in responses against DNA virus and bacteria pathogens.



Figure 2: TLR recognition of conserved molecular structures in bacteria, viruses, fungi and parasites.

Elie Metchnikoff identified the first innate immune effector cell, the macrophage, as a key player in cellular defense. Many others came after and today myeloid cells can be morphologically divided in two types: mononuclear and polymorphonuclear cells (or granulocytes composed by neutrophils, eosinophils, mast cells and basophils) that provide protection against bacteria and parasitic infections via release of toxic and inflammatory molecules ³³. The mononuclear phagocytes include the macrophages and their precursors, the monocytes, that reside allover the body regulating fibrosis and repair and contributing to immune surveillance and inflammation in a tissue-specific manner ^{34,35}. Also, the rich and essential group of dendritic cells (DCs) belongs to this myeloid family. While myeloid cells have long been considered essential for innate immunity, we realize now that cells of lymphoid origin, such as innate lymphoid cells (ILCs) and two peculiar populations of antigen-rearranged lymphocytes, $\gamma\delta T$ cells and B-1 cells, are likewise innate effectors due to their rapid and non-Ag specific activation. Given the importance of DCs and ILCs in innate immune responses, their biology will be more deeply explored in the following sections.

i. DENDRITIC CELLS

In 1976 Steinman & Cohn described a novel cell type in peripheral lymphoid organs with characteristic morphological 'dendrites' that they baptized as the 'dendritic' cell or DC ³⁶. Little did they know at that time that these "rare" DCs would be so critical for the proper functioning of the immune system and that DC biology would become a magnet for decades of intense immunology research. The description of other cells sharing immunogenic properties, phenotypic characteristics and morphology with DCs followed, building up the heterogeneous family that we know today (Figure 3). DCs are the professional antigen presenting cells (APCs) of the immune system that patrol secondary lymphoid tissues, most peripheral tissues and non-lymphoid organs. They are equipped with a molecular machinery to capture and process antigens, present them to T lymphocytes and provide co-stimulatory signals that shape immune responses. Numerous mouse studies have shown that DCs comprise a heterogeneous family with diverse ontogeny, locations, migration patterns and roles in immunity (Figure 3). The biology of human DCs, particularly in tissues, is less well understood and most of our knowledge on human DCs derives from blood studies. Some of the recent observations showed a high differentiation capacity of some DCs in circulation suggesting that within these there are precursors or immature DCs with different phenotype from tissue DCs ^{37–39}.

Myeloid DC-restricted precursors that exit the BM populate different tissues where they undergo differentiation *in situ* to give rise to two type of mature DCs: resident DCs that
will populate lymphoid tissues, and migratory DCs that will move from non-lymphoid tissues to the lymph nodes. The latter DC subset concomitantly up-regulates maturation markers upon migration and present tissue-derived antigens to T cells ^{40,41}.

Three subsets of DCs can be found in blood, spleen and tonsils within the lineage negative HLA-DR⁺ population: plasmacytoid DCs (pDCs, with plasma-like morphology instead of the typical dendritic shape) and two populations of 'classical' DC (cDCs) with abundant dendrites ⁴²⁻⁴⁴.

PLASMACYTOID DCs express a cluster of markers including CD123 (IL-3R), CD303 (BDCA-2) and CD304 (BDCA-4 or Neuropilin-1) (Figure 3). Recent work has revealed a rather multifaced identity of pDCs, being immunogenic or tolerogenic depending on the context ⁴⁵. pDCs play fundamental roles in the defense against viral infections through their potent type I interferon activity ⁴⁶. They can detect nucleic acids coming from virus, bacteria or death cells through TLR7 and TLR9 triggering the signaling cascade that ends up with the production of IFN-α, IFN-β and also IFN- λ ⁴⁷⁻⁴⁹.

In the steady state, thymic pDCs have a tolerogenic role by priming T_{regs} to produce IL-10 and TGF- β and regulating their proliferation and survival ^{50,51}. This function is accompanied by a low CD4⁺ stimulation capacity and MHC-II expression. Recent work showed that this type of tolerance involves a CCR9-dependent transport of peripheral Ags and subsequent deletion of Ag-reactive thymocytes ⁵². The tolerogenic machinery of pDCs has been reported as well in pathological conditions via TLR-independent activation (alternative stimulation). This results in the expression of indoleamine 2,3-dioxygenase (IDO) and inducible T cell co-stimulator ligand (ICOSL) that leads to induction of T_{reg} expansion and to an increase in IL-10 levels. ^{53–55}. This non-canonical pathway has been studied in the context of cancer, and is associated with a poor prognosis.

In contrast, pDCs become great drivers of both innate and adaptive responses following activation where they increase the expression HLA-DR and co-stimulatory molecules (CD40, CD80, CD86) and undergo a morphological change that converts them in functional APCs capable of presenting Ag to naïve CD4⁺ T cells and cross-priming CTLs ⁴⁷. Furthermore, activated pDCs production of IFN- α , IL-12 and IL-18 results in a massive activation of NK cells and CD8⁺ cells as well as Th1 polarization. Interestingly, some viral infections like HIV-1 lead to chronic immune activation that impairs pDC mediated IFN- α

production via enhanced CD40-CD40L interactions, evidencing the importance of this immune mechanism in antiviral response.

Moreover, pDCs can secrete other pro-inflammatory cytokines (such as IL-6 that can drive Th17 cell commitment) and chemokines (such as CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 3 (CCL3) and CCL4) that attract other immune cells to the sites of infection or inflammation. Given the importance of migration for the activity of these cells, it is not surprising that their function relies on the expression of several chemokine receptors, such as CXCR4 (development on the BM and migration to the spleen and LNs) and CCR2/CCR5 (blood recirculation) ^{56,57}.

CONVENTIONAL OR CLASSIC DCs are predominant in human blood and have been also reported in LNs and spleen as well as in non-lymphoid tissues including skin, liver, lung and gut. Two major cDC subsets can be identified by surface markers (Figure 3): CD1c⁺ DCs that co-express CD11b and high levels of CD11c and CD141⁺ cDCs that are lower for CD11c expression and negative for CD11b but express DNGR-1 (Clec9a). These cDC subsets differ in their TLR expression patterns: CD1c⁺ DCs express all TLRs except for TLR9 while CD141⁺ DCs have low expression of TLR1-2, TLR6 and TLR8 and high expression of TLR3 and TLR10 ^{58,59}.

In view of this TLR expression pattern, it is not surprising that *ex vivo* isolated blood $CD1c^+$ cDCs showed a broader cytokine expression capacity and in some cases also a more potent cytokine production (for IL-12, for example). Additionally it has been reported that TLR6 mediated CD1c⁺ DC production of IL-23 and shaping of Th17 response has a protective role on asthma ⁶⁰. By using human lung tissues and humanized mouse models, researchers found that specifically CD1c⁺ DCs are capable of driving CD103 expression on CD8⁺ cells and promote their accumulation in lung epithelia ⁶¹. In contrast, some unique properties have been attributed to CD141⁺ DCs, such as their expression of the chemokine receptor XCR1 that allows them to migrate in response to the XCL1, secreted by NK cells and CD8⁺ T cells ⁶².

Progress in the field of human DC research has been boosted in recent years thanks to in-depth proteomic and transcriptomic analyses and to the development of humanized mouse models that can be used to study the biology of these cells *in vivo*. Despite their phenotypic and functional differences, the two cDC subsets share characteristics including the capacity to uptake, process and present peptides to naïve CD4⁺ or CD8⁺ T cells. They are also similar in

terms of MHC-I expression ⁶³ and in stimulatory capacity of CD4⁺ T cells in mixed lymphocyte reactions (MLR). Naïve T cell differentiation and polarization is highly dependent on cDCs as evidenced by the immunodeficiency observed in individuals with IRF8 mutations (a transcription factor necessary for the development of mononuclear phagocytes) ⁶⁴. When referring to T-cell polarization the activity of the different subsets depends greatly on the TLRs and the cytokines implicated in the reaction. Many studies have focused on comparing the two cDC subsets in terms of capacity to elicit individual cytokine production under stimuli of different nature in distinct tissues. That detailed analysis is beyond the scope of this manuscript and has been reviewed elsewhere ⁴¹. In brief, both cDC subpopulations have been associated to Th1, Th2 and Th17 CD4⁺ polarization in various organs (LNs, lung and liver) with variable intensity depending on the nature of the stimulus.

The presentation of exogenous antigens on MHC-I molecules to CD8⁺ T cells that turns out in the initiation of the immune response is another fundamental characteristic of cDCs. *In vivo* CD1c⁺ DCs, CD141⁺ DCs and pDCs show equal intrinsic cross-presentation capacity although some may be specialized in certain types of antigens, like CD141⁺ DCs with necrotic cell-associated antigens ^{62,63}.

MONOCYTE-DERIVED DCs AND INFLAMMATORY DCs

Monocytes account for the majority of the phagocytic cells in human blood. They can be distinguished from DCs by the expression of CD14 but they share some of the other markers, like CD11c (Figure 3). Based on their expression of the low affinity Fc receptor CD16, they are classified in three subtypes: (1) classical CD14⁺⁺CD16⁻ (circulating blood guard that migrate to tissues upon inflammation), (2) intermediate CD14⁺⁺CD16⁺⁺ (main producer of inflammatory TNF- α *in situ*), and (3) non-classical CD14⁺⁺CD16⁺⁺ monocytes (IL-12, TNF- α and IL-1 β producers) ⁶⁵. Under homeostatic conditions monocytes patrol the body complementing the tissue surveillance of DCs but when inflammation occurs monocytes can differentiate into macrophages and dendritic cells, the so called monocytederived DCs or inflammatory DCs. These processes add more difficulties to the classification and distinction of "plastic" populations and discrete subsets. *In vitro*-generated moDCs using GM-CSF have been used as a model to study this cells ⁶⁶; however *in vivo* this "plastic" events seem to happen *in situ*. In mice, infDCs have demonstrated similar capacities as DCs, including production of pro-inflammatory cytokines, T cell priming and polarization of Th responses. However, the study of these cells in humans has been challenging and only a few reports claim the presence of infDCs with similar characteristics than those observed in mice in the context of atopic dermatitis, rheumatoid arthritis, and tumor ascites ^{67–69}. As such, our understanding of infDC function in humans is limited; humanized mice may provide a meaningful *in vivo* system to further study infDC biology.

	Function	Reference
1	Like TLR2	70,71,72
2	Upregulation of CCR7, IL-6, IL-10, IL-12p70, TNF- α , No INF- α	70,71,73,72
3	IFN-α (intermediate), IL-12p70 (high) IL-1β (CD1c+DCs), TNF-α, IL-6 CXCL-10/IP-10, CCL3, CCL4, CCL5 and IFN-β IFN-λ	70,71,72,63,58,74
4	Upregulation of CD80, CD86, CD83, CD40, CCR7 Secretion or upregulation of CCR7, IL-6, IL-8, IL-10, IL-12p70 No IFN-α response	71,73
5	Upregulation of CD80, CD86, CD83, CCR7 Secretion of TNF and IL-8 Upregulation of CCR7	70,71,72
6	Like TLR2 Upregulation of IL-23	70,71,72,60
7	Upregulation of CD40, CD80, CD86, CCR7 (in pDC) Very high IFN-α response (only in pDC) IL-12p70 response (only in cDC)	71,72
8	Like TLR7	70,71
9	Upregulation of CD40, CD80, CD86, CD83, HLA-DR, CCR7 Upregulation of IFN- α (very high), IFN- β (lower), IL-6, TNF- α (low), IL-8, IP-10 No IL-10 secretion	71,75
10	Upregulation of IL-8 (influenza infection)	76

Table 1: Main functions of toll like receptors in humans.

DENDRITIC CELL DEVELOPMENT

As previously mentioned, DCs derive from GMP in the bone marrow, circulate in the blood and home to the tissues throughout the body. However, the identification of human precursors restricted to one or several DC/monocytes/macrophage subsets and the understanding of the relationships between these precursors has been challenging due to the lack of suitable culture and *in vivo* systems. It has been suggested that the higher proliferation

capacity and lower expression of some maturation markers in circulating DCs indicated that those are, indeed, the precursors of DCs ³⁸. Recently, two studies described, both at bulk and single cell level, the hierarchy of myeloid precursors and proposed a developmental relationship between monocytes, cDCs and pDCs. Using a novel *in vitro* system and HIS mice, they proposed that GMP give rise to a monocyte-DC progenitor (MDP), which then differentiates, into a common DC progenitor (CDP) that produces the three main DC subsets. Furthermore, they report the identification of a circulating immediate DC precursor (hpre-cDC) that is restricted to the cDC subsets and expands in response to Flt3L (Figure 3) ^{39,77}.

The differentiation of HSCs via DC precursors to mature DCs is regulated by the environmental signals that includes cytokines, soluble factors and cell-to-cell contacts. Early attempts to generate DCs from hematopoietic precursors *in vitro* revealed that two cytokines were pivotal: GM-CSF and Flt3L. However, while the first gave rise to "monocyte-like" DCs, the second was unique in driving the generation of cDCs and pDCs.

The cytokine Flt3L (fms-like tyrosine kinase 3 ligand) is ubiquitously produced by stromal cells, endothelial cells and activated T cells. Its receptor, Flt3 (also called CD135 and Flk2) is strongly expressed by early hematopoietic precursors (GMP, CLP) and is maintained on dendritic cells lineage restricted precursors and mature pDC and cDCs (Figure 3) ⁷⁸. Accordingly, ablation of Flt3 is correlated with loss of DC differentiation potential ⁷⁹. On the other hand, Flt3L administrated in mice and in humans leads to a drastic systemic expansion of both pDCs and cDCs ^{80,81}. Therefore Flt3L is not only important for the development but also the homeostasis (survival, proliferation) of peripheral DCs ⁷⁸.



Figure 3: Schematic view of human dendritic cell development The main surface markers for each subset are shown as well as the cytokines they produce (in green). GMDP: granulocyte, monocyte and dendritic cell (DC) progenitor; CDP: common DC progenitor; MDP: monocyte-DC progenitor; pre-cDC: commited precursor of classical DCs.

ii. INNATE LYMPHOID CELLS

Innate lymphoid cells are a recently identified family of innate effectors. Two of its founding members: the natural killer cell (NK cell) and the lymphoid tissue inducer cell (LTi cell) have been known and studied for many years but the identification of new subsets with similar characteristics prompted their "clustering" as a distinct but heterogeneous effector lineage. ILCs lack recombination activating gene (RAG)-dependent rearranged antigen receptors and share a lymphoid cell morphology. They can be identified by the lack of lineage markers (CD3/CD19/CD14/CD33...) but express CD7 and CD127 (except for CD56^{dim} NK cells). Interestingly ILC effector functions and transcription factor dependency mirror those observed in T cell subsets prompting the speculation that ILCs are 'innate' counterparts of differentiated T helper and cytotoxic lymphocytes (Figure 4). Accordingly, we can distinguish three broad groups of ILCs paralleling the three major axis of T cell specialization. ILC1s depend on the transcription factor T-BET and can be subdivided into cytotoxic ILC1 or NK cells (like CTLs) and ILC1s that preferentially produce type 1 cytokines (like Th1 cells). Likewise, ILC2s and ILC3s are reminiscent of Th2 and Th17/Th22

cells in that they depended on GATA-3 or $ROR\gamma t$, respectively, and they elicit the corresponding cytokine response (see in detail below). NK cells appear distinct from other 'helper' ILCs in that they have different requirements for cytokines and transcription factors (reviewed in ⁸²).



Figure 4: Innate lymphoid cells closely reseamble T cel subsets. *(Adapted from Goldberg et al. 2015)*

TYPE 1 ILCS

For almost forty years, NK cells have been considered the prototypic (and in some cases only) innate source of type 1 cytokines during the early phases of the immune response. Several studies in the last decade, first in mice and then in humans, have identified additional phenotypically diverse ILC1 subsets (particularly inside the tissues) that provide innate sources for IFN- γ . Before discussing these different ILC1 subsets, I will briefly present some of the general features that characterize human NK cells.

Natural Killer Cells

Natural killer cells owe their name to the unique ability to kill transformed or virus infected cells without prior sensitization ⁸³⁻⁸⁵. Although originally described by this natural capacity to kill, NK cells are also major sources of type 1 cytokines, notably IFN- γ , TNF- α and GM-CSF, and to a lesser extent other cytokines and chemokines (MIP-1 α , MIP-1 β and RANTES)⁸⁶. Even before the discovery of additional ILC1 subsets, a high degree of heterogeneity was already appreciated among NK cells by virtue of the distinct lineage differentiation stages, the variety of effector functions and their tissue localization. In humans two major subsets can be distinguished in the circulation by their expression of the adhesion molecule CD56, namely CD56^{bright} and CD56^{dim} subsets (Figure 5). CD56^{bright} cells are considered to give rise to the fully mature CD56^{dim} cells, based on the order of appearance in HSC transplantation experiments and cytokine driven models of in vitro NK cell differentiation, and also to the length of the telomeres observed in the two subsets ^{87,88}. CD56^{dim} cells express the low affinity Fc receptor CD16 (responsible for the antibodymediated cell cytotoxicity, ADCC) and account for 90% of the peripheral blood NK cells. This subset is highly cytotoxic thanks to the cargo of effector molecules, granzymes, perforin and Fas ligand, contained in secretory lysosomes that provide them with a characteristic granular morphology ⁸⁹. The CD56^{bright} subset, contrarily, has a low-density expression of CD16 but exhibits a high potential for cytokine production under monokine or target cell stimulation. These cells are also hyper-responsive to IL-2 due to the expression of the highaffinity heterotrimeric IL-2 receptor thus showing a higher proliferative potential and the capacity to acquire potent lytic function. CD56^{bright} are better at penetrating the secondary lymphoid tissues thus accounting for the majority of the NK cells in lymph nodes or decidua, whereas in lung NK cells have a very differentiated profile.



Figure 5: Identification of human peripheral blood NK cells by flow cytometry (A) and general phenotype and effector funciton of CD56^{bright} and CD56^{dim} NK cell subsets (From Cooper et al, 2001) (B).

Given the modulation of CD56 and CD16 expression after activation, this dual classification of NK cells may be inaccurate in the context of tissues. For example, in the decidual tissue NK cells are CD56^{superbright} accounting for around 70% of the lymphocytes during the first months of the pregnancy where they interact with the trophoblast and other immune cells regulating arterial remodeling and placenta development ⁹⁰. These cells, called uterine NK cells (uNK) are considered a distinct NK population as they display some other characteristics different from pNK cells like a particular pattern of KIR expression and the over-expression of CD9, galectin, alpha-1 integrin, as well as other adhesion molecules ^{91,92}. Interestingly, these NK cells are able to induce T_{reg} cells in vitro indicating a role in maternal tolerance ^{93,94}. Even within the CD56^{dim} subset one can find diverse "stages" with particular phenotypic and functional properties throughout their final differentiation ^{95,96}. This progression from CD56^{dim} to terminal differentiated cells is accompanied by the progressive loss of the proliferation capacity and the acquisition of more efficient cytolytic activity. Phenotypically, down-regulation of CD94 and NKG2A and up-regulation of CD16, KIR and perforin expression characterize this process that terminates with a CD57-expressing highly cytotoxic mature population ⁹⁷. Interestingly, cytomegalovirus infection (CMV) leads to selective expansion of this terminal mature subset, the drastic up-regulation of NKG2C expression ^{98,99} and the display of certain hallmarks of adaptive immunity ¹⁰⁰.

Despite their rapid and potent response upon viral infection or transformation, NK cells are efficiently rendered 'self-tolerant' thereby avoiding potential autoimmunity. This is achieved by an 'education' process generating an array of activating and inhibitory receptors that maintain the NK cells on alert while tolerating "self" ¹⁰¹. Additionally, NK cells constitutively express monokine receptors that allow them to respond strongly to cytokines such as IFN- α , IL-2, IL-12, IL-15 and IL-18, which are produced by surrounding cells like monocytes and dendritic cells ¹⁰². It is therefore the combined effects of cytokines and receptor-ligand engagement that condition NK cell responses ¹⁰³.

The training of a self-tolerant NK cell is dependent on the signaling through ITIM bearing inhibitory receptors ¹⁰⁴⁻¹⁰⁶ and takes place by a mechanism not yet agreed by the experts. Four main mechanisms have been suggested (Figure 6): "the arming" hypothesis establishes that NK cells are initially unresponsive and the encounter of MHC-I by inhibitory receptors during development licenses or "arms" them (Figure 6a) ¹⁰⁴. The opposite possibility has also been suggested, that is the "disarming" hypothesis, stating that initially responsive NK cells would become anergic after chronical stimulation unless engaged by MHC-I specific to its inhibitory receptors (Figure 6b) ¹⁰⁷. A third mechanism refers to as "cisinteraction model" relies on the ability of KIR receptors to bind to MHC-I molecules in cis and is based on the observation that KIR receptors can transmit inhibitory signals even in the absence of ligand interaction, but it is unclear whether this model can be applied to all KIR receptors (Figure 6c) ¹⁰⁸. Finally the "rheostat model" proposes that NK cell reactivity is tuned by the number and degree of affinity of self-MHC-I inhibitory receptors carried by a cell (Figure 6d) ^{107,109}. As such, NK cells experience a quantitative functional adaptation that generates MHC-I educated NK cells responding efficiently to stimulation and responding as well to aberrant cells that have lost MHC-I (missing-self recognition)¹¹⁰.



Figure 6: Models of MHC-I mediated education of NK cells. (From Höglund et al. 2010)

Other ITIM bearing receptors contribute to maintaining NK cells on control, including NKG2A that binds the non-classical MHC-I molecule HLA-E, and LIR-1 that engages both classical MHC-I and the non-classical HLA-G ^{111,112}. Additionally, non MHC-I ligands can bind ITIM-containing receptors, this is the case of KLRG1 that binds cadherins and participate in tumor invasion and metastasis detection ^{113,114}, and CD161 that recognizes LLT1 (osteoclast inhibitory lectin or CLEC2D, expressed by DCs and B cells) thus regulating NK cell activation (Figure 7) ¹¹⁵.

Unlike T cells, allogeneic NK cell transplantation leads to graft-versus-leukemia responses without causing graft-versus-host disease (GVHD) ¹¹⁶. This suggests that NK cells are not pre-wired to kill in mismatched contexts but other positive stimuli contribute to triggering the response. This positive recognition of « altered-self » or pathogen derived molecules is carried out by activating receptors that express or not ITAM-carrying adaptors. Among those that signal through ITAM are the activating KIRs, such as KIR2DS and KIR3DS, as well as the heterodimers CD94-NKG2C and CD94-NKG2E ^{117,118}. Intriguingly, both inhibitory NKG2A and activating NKG2C heterodimers bind to the same ligand suggesting that a more sophisticated mechanism of NK regulation might exist. ITAM-independent receptors include NKG2D whose ligands are MICA-B and ULBPs, induced in stressed cells (transformed or virus infected) ^{119,120} and also the family of SLAM-related receptor (SRRs), notably 2B4 greatly contribute to the regulation of NK cell function when binding CD48-expressing cells ¹²¹ (Figure 7).

An additional family of activating receptors, the natural cytotoxicity receptors (NCRs) –NKp46 (NCR1), NKp44 (NCR2), NKp30 (NCR3)- are expressed in activated and resting NK cells (Figure 7). The role of this family of receptors in human NK cell-mediated killing was demonstrated by Moretta and colleagues in culture experiments containing human NK clones and NK-susceptible tumor cells. Addition of anti-NCR monoclonal antibodies directed against individual NCRs resulted in partial inhibition of cytotoxicity whereas the combined use of all three mAbs strongly abrogated cytolysis ¹²². This work suggested that NCRs cooperate in target cell recognition and killing and that the extent of that cooperation depends on the density of the ligands on target cells. Furthermore, experiments in mouse showing the reduction of the tumor cell lysis by NK cells lacking one or more NCRs reflect their high importance in *in vivo* tumor surveillance ¹²³. Several studies have also reported exogenous ligands for NCRs (reviewed in ¹²⁴), notably pp65 of HCMV for NKp30 and viral HA for

NKp44 and NKp46 further evidencing the protagonist role of these receptors in NK cell function ^{125–127}

The outcome of the receptor-ligand binding not only depends on the identity of the receptor itself but also by the adaptor proteins associated to the cytoplasmic domains. As such, the 2B4 NK receptor (also called CD244) behaves as a multifunctional receptor and the outcome of its engagement seems to depend on the stage of NK cell maturation and activation. Ligation of 2B4 with a specific antibody or with CD48-expressing cells lead to NK activation while in patients deficient for SAP (a 2B4 adaptor protein) that engagement results in inhibition of NK cell function ^{115,128}

Tens of other activating NK receptors have been identified as well as co-stimulatory receptors that act synergistically ensuring the activation of NK cells upon "unhealthy" circumstances and not otherwise and modulating the intensity and type of immune response. These include DNAM-1, NKR-P1, PILR, LFA-1, CD2 and others depicted in Figure 7. A more exhaustive description of these receptors can be found in several excellent reviews published over the last decades ^{124,129,130}.



Figure 7: Activating and inhibitory receptors of human NK cells. (Adapted from Nature Reviews Poster, Vivier&Ugolini)

Surface NK cell receptors serve as landmarks that dissect the stepwise differentiation of HSCs to mature NK cells. Human NK cells can be detected in fetal liver from week 6 of gestation and in BM, lymph nodes, spleen, lung and intestine during the second trimester ¹³¹. In adults, early stages of NK cell development take place in the BM whereas some

multipotent precursors may exit to extramedullary tissues where molecular signals drive their final differentiation ^{132,133}. This process is regulated by the integrated influence of soluble factors and transcriptional regulators that modulate lineage-specific gene expression^{134,87,135}. Restricted NK precursors, including pro-NK and pre-NK (CD34⁺CD45RA⁺CD117⁺ CD94⁻CD122⁺) cells retain some multi-lineage potential, suggesting that these populations were not fully NK cell committed. Recently, a refined NK-restricted progenitor (rNKP) downstream of CLP has been defined with exclusive potential to the NK cell lineage after *in vitro* single-cell and *in vivo* transfer experiments (Figure 8)¹³⁶. These cells, identified by the cell surface expression profile Lin⁻CD34⁺CD38⁺CD123⁻CD45RA⁺CD7⁺ CD10⁺CD127⁻ are localized in fetal liver (FL), BM, cord blood and adult tonsils. Other upstream NK precursors that retain the capacity to differentiate not only in NK cells but also in helper ILCs have been proposed recently and will be discussed later.



Figure 8: Model of early NK cell development. (From Renoux et al., 2015)

NK cells are present in most compartments of the human body, from primary and secondary lymphoid tissues to peripheral organs. These NK cells have two origins, some of them are PB-NK cells that patrol the circulation and get recruited to the tissues, particularly upon pathologic insults to exert locally an immune response. In contrast, a second NK cell subset resides permanently in tissues, and shows phenotypic and function specialization shaped by the local environment. This tissue resident NK cell (trNK) subpopulations have been associated to the uterus (discussed earlier), skin, thymus, liver, intestines and salivary gland (at least in mice). In addition to their capacity to respond more rapidly, trNK share some attributes previously described for human and mouse resident-T cells. trNK express

CD69, which inhibits surface expression of sphingosine-1-phosphate receptor 1 (S1PR1) thereby promoting their retention in tissues ¹³⁷; CD69 is absent from PB-NK cells but is expressed by skin, liver, uterus, lymph nodes and intestine human NK cells ^{138–142}. Additionally, other hallmarks for tissue residency, including the expression of CD103 (that binds E-cadherin in epithelial cells) and/or CD49a (that promotes homing to non-lymphoid tissues), the tissue homing receptors CXCR6 or CCR5, and the lack of the adhesion molecule CD62L have been ascribed to some human NK cells in those organs ¹⁴³. With the establishment of ILCs as a family that distinguishes between cytotoxic (mostly recirculating) NK cells and helper (mostly resident in steady-state) ILCs some of the trNK cells have been re-classified as non-NK ILC1s ¹⁴⁴.

Helper ILC1s

The discovery in 2006 of IFN- γ -producing cells that, unlike NK cells, depend on the transcription factor Gata-3 and express IL-7 receptor (CD127) in the thymus suggested diversity in innate lymphoid developmental pathways. We know now that these two characteristics are shared by 'helper' ILC subsets thus placing thymic NK cells as a group 1 ILC member ^{145,146}.

To date four different ILC1 populations have been identified in mice by virtue of their phenotype and dependency on T-bet and Eomes transcription factors. However these subsets have overlapping yet distinct phenotypes and functions (in an organ-dependent fashion) which makes an unambiguous ILC1 classification a challenge. The limited access to organs and the lack of *in vivo* genetic tracing or modifying technologies in humans further complicates this endeavor. As such, human ILC1 are largely defined based the absence of other known markers for NK cells, ILC2s or ILC3s. These include the high expression of CD127 and CD161 and the absence of CD56, CD94, NKp44, NKp46, c-kit, granzymes and perforin. Human ILC1 produce IFN- γ but not IL-13, IL-17 or IL-22 and are T-BET-positive but lack ROR γ t, GATA-3 or ROR α ¹⁴⁷. Given their localization, the post-birth emergence and the fact that they expand in the context of Crohn's disease these ILC1s are likely involved in the early immune response and maintenance of homeostasis at mucosal barriers (Figure 10). Indeed, mouse models of innate inflammation showed that Ab-blockade of IFN- γ production ameliorated the local and systemic inflammation related pathology ¹⁴⁸.

In the interface between the phenotypic definition of ILC1 and NK cells, intraepithelial ILC1 patrol the gastrointestinal mucosa watching out for danger signal from epithelial and myeloid cells. Fuchs and colleagues described this IEL population both in the

human tonsils and intestine (NKp44⁺CD103⁺) and in mice (NKp46⁺NK1.1⁺CD160⁺), where they develop independently of IL-15¹⁴⁹. These ILC1s express low levels of CD127, CD56, NKp44 and CD94 and produce IFN- γ in response to IL-12 and IL-15 (Figure 9). Interestingly, they showed several hallmarks of TGF- β imprinting, such as expression of CD103 and CD9, characteristics also observed in uNKs. Like cNK cells they depend on T-BET and EOMES but develop independently of IL-15R α (al least in mouse) pointing to a dependency on other cytokines such as IL-7 or IL-2¹⁴⁹. Recently, a study on the PB-ILC repertoire reported CD127⁺CRTh2⁻CD117⁻ cells expressing some T cell markers (cytoplasmic CD3, CD4, CD8, CD27 and CD28) but lacking $\alpha\beta$ -TCR, $\gamma\delta$ -TCR in the cytoplasm and CD3-TCR complexes in the cell surface ¹⁵⁰. Single-cell RNA sequencing of tonsil CD127⁺ ILCs was also able to detect this population albeit with a low T-BET expression and high expression of some T cell-associated genes, raising the need for further studies that corroborate the real identity of this intriguing population ¹⁵¹.

The identification in mice of a IFN-γ-producing population in liver distinct from NK cells that displays some adaptive-like features prompted the search for a human counterpart ¹⁵². As such, CD49a⁺ ILC1s that depend on T-BET but not on EOMES were found in human liver but not in the hepatic venous or in peripheral blood. Moreover, they express KIR and NKG2C and are CD56^{bright} but are low for CD16, CD57 and perforin. Consequently, they can express high levels of pro-inflammatory cytokines but degranulate poorly ¹³⁹. However, the highly phenotypic similarity with conventional NK cells and their lack of IL-7R (common to all the other hILCs) suggests that these cells may be more like NK cells than 'helper' ILC1. A recent report has argued the existence of this population and has proposed instead a novel subpopulation of CD49e⁻ liver resident NK cells by using cytometry by time-of-flight (CyTOF) and humanized mice.

In mice an additional ILC1 population has been described in salivary glands that resembles liver ILC1 in the expression of CD49a and TRAIL but, like cNK cells, are DX5⁺ and rely on Eomes. Additionally, SG ILC1s are poor producers of IFN γ contrasting with ILC1 hallmark characteristics ^{153,154}. It remains to be investigated the presence and physiological importance of this population in man. A very recent report described for the first time in human lung the presence of ILC1 but further phenotyping and functional studies are needed to confirm their identity ¹⁵⁵. Likewise, an uterine ILC1 population has been mentioned recently capable of producing IFN- γ but different of the aforementioned uNK cell

in that they do not express EOMES and they are CD49a-positive, thus resembling the cells mentioned earlier in mucosal tissues ¹⁵⁶.

It is essential for the accurate identification of these populations to keep in mind that some of the aforementioned markers maybe modulated in the context of inflammation or activation, like in the case of CD49a¹⁵⁷. Also, fate-mapping experiments in mice and *in vitro* assays in humans revealed transdifferentiation of ILC2s and ILC3s into IFN- γ -producing cells by the upregulation of T-bet and downregulation of Gata-3 or ROR γ t further complicating the *in vivo* identification of ILC subsets in humans^{158–162}.



Figure 9: ILC subsets and their signature cytokines. ILCs are activated by injured or infected cell signals and respond by releasing signature cytokines that promote important effector and regulatory responses. *(From Eberl et al. 2015)*

TYPE 2 ILCS

ILC2s are defined by their innate capacity to produce type 2 cytokines, mainly IL-13, IL-5, IL-9 and IL-4, as well as amphiregulin (Figure 9). These soluble factors have a dual effect being either protective to the host or capable of exerting pathogenic activity. IL-13 promotes resistance to large extracellular parasites, such as helminthes, by increasing mucus production and muscle contractility, favoring epithelial cell turnover and macrophage activation. Furthermore, type 2 immunity has proven protective in a range of autoimmune diseases (arthritis, multiple sclerosis or Crohn's disease) by the suppression of type-1 driven

inflammation (notably by TSLP) and eosinophilia (IL-4) and is involved in tissue repair (amphiregulin) and in the metabolic homeostasis (Figure 9).

Like the other ILCs, type 2 ILCs sense their environment and get activated by cytokines and alarmins produced by myeloid and stromal cells including IL-33, IL-25, TSLP, IL-2, IL-4, IL-7, TL1A, prostaglandin D_2 and leukotriene D_4 ^{163–168}. They are capable of sensing these molecules thanks to the array of surface receptors expressed that also help us define their phenotype. Human ILC2s can be identified by expression of CD7, the common-gamma chain (CD132), CD127 and CD25 (rendering them responsive to IL-7 and IL-2), as well as CRTh2 (prostaglandin D2 receptor), CD161 and CD11a and to some extent also CCR6, CD117, KLRG1 ^{160,169}. ILC2s have been found in human tissues including lung, skin, nasal mucosa, adipose tissue and gut, and there their phenotype is slightly different than in the circulation, with essentially all ILC2s expressing CD90, CD44, ICOS and ST2 (IL-33R) as well as IL-25 receptor (IL-17BR) ^{163,170,171} highlighting the influence of environmental tissue adaptations. Moreover, ILC2 can be identified by the expression of the master transcription factor GATA-3 as well as the dependency on ROR α and TCF-1 for their development from the CLP ^{172–175}.

In mouse an IL-25 responsive ILC2 subset has been characterized expressing high levels of KLGR1 and IL-25R, denoted "inflammatory ILC2" ¹⁷⁶. This population can express RORγt and produce IL-17 to fight *Candida Albicans* infection. It is unclear whether inflammatory ILC2 exist in humans and to what extent plasticity drives the generation of this subset.

The first functional role found in ILC2s was the resistance to worm infections in mouse intestine; a process that is dependent on IL-25 and IL-33 mediated activation of ILC2s. This occurs through the production of IL-13 and the consequent goblet and tuft cell hyperplasia, muscle contraction and activation of "type 2" macrophages. Concomitantly tuft cells produce IL-25 thus creating a positive loop that amplifies the response resulting in worm expulsion. In children ILC2 have also been found to inversely correlate with the presence of helminthic infection and with infection intensity, with the youngest patient group (children aged 6-9) showing the strongest correlation ¹⁷⁷. However other studies of similar pathologies have claim contradictory results advocating for further data before drawing conclusions in that regard ¹⁷⁸. Furthermore, ILC2s are capable of secreting amphiregulin to promote tissue epithelial repair after influenza infection and possible other tissue disrupting disorders (Figure 10) ¹⁷⁰.

However, when produced in excess ILC2-derived cytokines lead to allergic disorders, such as asthma, atopic dermatitis and multiple allergies (Figure 10) ^{179,180}. Several allergens have been used in mice to recapitulate lung or skin inflammation, a process mediated by IL-25 and IL-33 as well as by basophil-derived IL-4. The involvement of ILC2s in human atopic diseases have been confirmed by the observation of increased activated ILC2s in skin lesion and nasal polyps of patients with atopic dermatitis and nasal polyps ^{181,182}. Immunopathology provoked by an excessive or prolonged ILC2 response can be counteracted by type I and II interferons and IL-27 ¹⁸³ as well as by the expansion of T_{reg} cells.

The observation that ILC2 secrete cytokines constitutively in steady state suggests that these cells are important not only for immune responses but also to maintain the tissue homeostasis. Indications of these homeostatic roles are the correlation of IL-5-mediated eosinophil control with the circadian rhythm ¹⁸⁴ or the maintenance of the epithelial-stem-cell compartment and regulation of tuft-cell development ¹⁸⁵.

TYPE 3 ILCS

LTi cells were initially described as CD3⁻CD4⁺ cells essential for the formation of lymphoid tissues¹⁸⁶. We now appreciate that LTi cells represent a prototypical members of the heterogeneous group 3 ILC that exist in both in mouse and in humans and express the signature transcription factor Roryt.

CCR6+ LTi cells express lymphotoxin- $\alpha_1\beta_2$ that during mouse fetal development binds to LT β R on stroma and endothelial cells regulating the formation of lymphoid structures, such as lymph nodes, cryptopatches and Peyer's patches ¹⁸⁷. LTi cells have been reported also in human mesenteric LNs and spleen during the first months of pregnancy ^{188,189}. In adults this interaction is involved in the positive loop that potentiates the production of IL-22 through the release of IL-23 by DCs in the context of bacterial infection ¹⁹⁰ as well as in promoting immunoglobulin production by B cells ¹⁹¹. Contrarily to mouse, human LTi cells do not express CD4 but are CD161⁺CD7⁺ and they are able to produce IL-22 and IL-17.

Adult human ILC3s are generally defined by their capacity to respond to IL-23 and IL1- β by secreting IL-22 (Figure 9). They are generally divided into two groups, those expressing NKp44 and/or NKp46 (NCR+) and those negative for NCRs. In human, ILC3s have been described in tonsils, spleen, endometrium, decidua, skin and lung ¹⁹¹⁻¹⁹⁶. The few published data on human specimens show a broader and variable cytokine production capacity depending on the tissue and context analyzed. In tonsils, this population is sensitive

not only to cytokine stimulus but also NKp44-mediated signaling, and it is capable of producing IL-2, TNF, GM-CSF and LIF in addition to IL-22 ¹⁹⁶. Also, in inflamed conditions of Crohn's disease, NCR-ILC3s are able to produce IL-17, which could likely contribute to the severity of the disease ¹⁹⁷. Furthermore, GM-CSF derived from splenic ILC3s can promote survival and differentiation of MZ B cells ¹⁹¹. Recently ILC3s subsets have been found in human endometrium and decidua with NCR+ ILC3 and LT_i-like cells producing pro-inflammatory cytokines such as IL-8, IL-22, IL-17A, TNF and IFN-γ, contributing to antimicrobial defenses during pregnancy and vessel remodeling ¹⁹³.

As repeatedly demonstrated in sepsis models using *E. coli*, ILC3s are important in the early clearance and resistance to bacterial infection ^{198,199}. Binding of IL-22 to epithelial cells leads to a STAT3 mediated signaling that concludes with increased cell proliferation, secretion of antimicrobial peptides and the fucosylation of epithelial cells ²⁰⁰. In these models, the early IL-22 mediated response of ILC3s is critical, and activation is dependent on the IL-23 released by DCs and mononuclear phagocytes ²⁰¹. However, recent studies have questioned ILC3 roles based on possible functional redundancy with Th17 cells ²⁰². To date no mouse model has been developed that specifically depletes ILC3s without affecting other cell population that could resolve this question.

Detrimental inflammatory disorders have been related to ILC3s both in mouse models of colitis and in patients of Crohn's disease (Figure 10)^{197,201}. This is likely due to the loss of commensal bacteria T cell tolerance to which ILC3s contribute through MHC-II peptide presentation. Indeed, analysis of Crohn's disease patients evidenced their role in intestinal tolerance regulation, with MHC-II expression being inversely correlated with the number of Th17 cells²⁰³. Also, clinical data from individuals with psoriasis vulgaris pointed to a role of ILC3 in skin inflammation with accumulation of these cells in skin and blood (Figure 10)²⁰⁴. However, the lack of an appropriate mouse model to study human ILCs has hampered so far the elucidation of the underlying mechanisms. Patients with lung inflammatory conditions such as COPD, also showed higher numbers of NCR- ILC3s and increase IL-17 but the connection and mechanism remains unknown²⁰⁵. A very recent paper showed the direct inverse link of ILC numbers in blood and HIV infection that could only be avoid when patients were ART-treated in the acute phase of the infection²⁰⁶.

Both in mice and in humans ILC3s have been associated with anti-tumor responses (Figure 10). In non-small-cell lung cancers NCR+ ILC3s are present in the lymphoid infiltrate of tertiary lymphoid structures found in these cancers and are associated with

favorable clinical outcomes ¹⁹⁵. Intriguingly, high IL-22 signaling in acute inflammation has been related with increased tumor susceptibility and tumor growth after several induced mouse tumor models ²⁰⁷. Nevertheless, the mechanisms and relevance in the human setting remains elusive.

Like ILC2s, type 3 ILCs are essential in the regulation of homeostasis in the interface with the microbiota, in this case by the IL-22 driven proliferation and survival of intestinal epithelial cells as well as the GM-CSF mediated maintenance of the myeloid compartment ^{208,209}. Finally, mouse experiments have revealed another interesting role of ILC3s in the protection against ionizing radiation, through maintenance of the intestinal stem-cell pool and thymic epithelium ²¹⁰. Recent data on acute myeloid leukemia patients undergoing preconditioning therapy before the HSCT confirmed those results, with NCR+ ILC3 expansion and homing to gut and skin after chemotherapy ²¹¹.

Interestingly, ILC2-promoting cytokines, such as IL-25, inhibit IL-22 production by ILC3s thus establishing a dynamic equilibrium ready to respond upon internal signals or microbial threats (Figure 10). A remarkable characteristic of some (possibly all) ILCs is their capacity to adapt to environmental cues or trans-differentiate, like NCR- ILC3s that turn into NCR⁺ ILCs in the presence of IL-1 β and IL-23 and into ILC1 when IL-12 is present ¹⁴⁷. Signaling through IL-1 β , IL-23 and retinoic acid can promote ILC1 trans-differentiation back to ILC3 ¹⁵⁸. Plasticity can create compensatory effects on the numbers of certain ILCs at the expense of other types especially in the context of inflammation.



Figure 10: ILC role on inflammation and tissue repair. ILC dysfunction has been found in several pathologic conditions and has been related to the direct or indirect effect of their signature cytokines. (*From Artis&Spits 2015*)

iii. GAMMA-DELTA T CELLS

 $\gamma\delta$ - and $\alpha\beta$ -T cells derive from a common early precursor in the thymus where they both undergo TCR RAG-mediated V(D)J somatic recombination. Despite the developmental similarities, our understanding of the biology of $\gamma\delta$ -T cells has lagged behind, in part due to the difficulties to identify the antigens these cells recognize. In humans $\gamma\delta$ -T cells have a small repertoire of V gene segments and thus a narrow specificity. Most frequently the γ chains used are V γ 2 to V γ 11 and for the δ segments only V δ 1, V δ 2 and V δ 3 are commonly rearranged, thus generating a certain degree of heterogeneity. The restricted variability led to the initial hypothesis that these cells recognize conserved self-proteins ²¹². However recent studies revealed some of these specificities uncovering a high immuno-therapeutic potential ²¹³. The two most important antigen families are phosphorylated prenyl metabolites, lipids presented by CD1 proteins, and cell stress-associated molecules that get upregulated after transformation or infection, such as MICA and MICB (Figure 11). Indeed already a decade ago, mouse studies uncovered a critical role of $\gamma\delta$ -T cells in skin cancer immunosurveillance ²¹⁴ and more recently a meta-analysis of 18000 cancer patients disclosed the intratumoral presence of this cell population as the most significant signature of favorable prognosis ²¹⁵. During the development in the murine system different subsets of $\gamma\delta$ -T cells target specific tissues, such as skin, lung, uterus, vagina and tongue and integrate the intraepithelial lymphocyte population performing very specialized roles ²¹⁶. This is the case of the so-called dendritic epidermal V γ 3V δ 1 T cells (DETCs) that sense skin injury and regulate the local immune response ²¹⁷.

V δ 1⁺ T cells account for half of the total blood $\gamma\delta$ -T cells at birth but their frequency decreases with age as these cells home to specific sites, notably to epithelial tissue and particularly in the intestine ²¹⁸. In humans, $\gamma\delta$ -T cells recognize "MHC-like" molecules, such as the CD1 family or stress-induced peptides. CD1 family, including the best characterized CD1c and CD1d, are molecules capable of presenting both foreign and self-lipid antigens ²¹⁹. Although the precise identity of these ligands is largely unknown, several studies demonstrated the fundamental role of the "non-V δ 2" $\gamma\delta$ -T cells in maintaining the homeostasis of the local microenvironment, in promoting wound healing and removal of distressed or transformed epithelial cells as well as lessening down the local inflammation ²²⁰⁻²²². V δ 1 pairs preferably with V γ 4 and V γ 5 and to a lower extent to V γ 9, indicating the existence of several antigen specificities ²²³. These V δ 1 T cells are also recruited to the

maternal/fetal interface during pregnancy and increase in numbers and activation status probably leading to the induction of tolerance ²²⁴.

The majority of $\gamma\delta$ -T cells in adult human blood bear a V δ 2 TCR (1-5% of total T cells), usually paired to V γ 9 chain. These V γ 9V δ 2 T cells recognize non-peptidic phosphorylated antigens, like isopentyl pyrophosphate (IPP), a metabolite of the cholesterol pathway and other metabolic intermediates that get accumulated in the context of malignancy or cellular stress ²²⁵. Other stress-induced ligands have been investigated, such as the ATP synthase that, after translocation into the membrane, drive the secretion of inflammatory cytokines by $\gamma\delta$ -T cells, likely by phospho-antigen presentation ²²⁶. Furthermore, it is now well accepted that the butyrophilin 3A1 (CD277) is a target for V γ 9V δ 2-T cells but the molecular mechanisms of that interaction remains obscure ²²⁷. The fact that these pAgreactive $\gamma\delta$ -T cells exist only in primates point to a non-redundant function in the stress surveillance needs that appeared late in evolution.



Figure 11: Receptor-ligand interactions mediating tumor cell recognition by gamma delta ($\gamma\delta$) T cells (From Silva-Santos et al, 2015)

Altogether, $\gamma\delta$ -T cells show a very particular tissue-specific localization of oligoclonal subpopulations sharing the same TCR chains, making up 20-50% of the total lymphoid population in intestine and dermis ²²⁸. Research over the last years has revealed a certain phenotypic and functional heterogeneity within this population. Recent studies point to a preferential type I response by $\gamma\delta$ -T cells and even in the absence of TCR stimulation

they are capable of responding to exogenous IL-2 or IL-15 by producing IFN- γ and killing tumor cell lines (Figure 12) ²²⁹.Tumor cell recognition and activation occurs through engagement of TCR and/or natural killer receptors, including NKG2D, NKp30 and NKp44 (Figure 11). This activation leads to the aforementioned cytokine response but also to cytotoxic activities, including the release of perforin and granzymes and the expression of CD95 ligand and TRAIL ²³⁰.



Figure 12: Antitumor versus protumor roles of $\gamma \delta$ -T cells in the mouse. (From Silva-Santos et al. 2015)

 $\gamma\delta$ -T cytokine secretion profile goes beyond type I response with cases of allergic asthma patients showing $\gamma\delta$ -T-derived IL-4 in the bronchoalveolar lavage fluid ²³¹. Other particular cytokines are expressed by $\gamma\delta$ -T cells, such as keratinocyte growth factors and connective tissue growth factor (CTGF), showing important functions in control of epithelial integrity. Their immunosuppressive capacity has also been demonstrated by the ability to produce II-10 and control CD8⁺ T cell expansion and function ²³².

Paradoxically, one report has also attributed a pro-tumor role for IL-17 producing $\gamma\delta$ -T cells by the expansion of myeloid-derived suppressor cells in human colorectal carcinoma (Figure 12)²¹³. Of note, these cells were also capable of producing IL-8, TNF and GM-CSF and have been observed, as well, in other cases with severe inflammatory conditions, such as

children with bacterial meningitis and non-melanoma skin cancer patients ²²⁹. These observations are thus far outbalanced by the reports showing an anti-tumor activity so efforts are being made to boost and apply $\gamma\delta$ -T cells as therapeutic alternatives for cancer patients.

Given their proficiency as stress sensors and cytokine producers it is not unexpected that these cells play important roles during viral infection. Indeed, the scarce studies that begin now to be known point to such role in HIV patients where $\gamma\delta$ -T cells are drastically reduced and they do not recover even after antiretroviral treatment, when CD4⁺ T cells are normalized ²³³.

Interestingly, $\gamma\delta$ -T cells not only can act as effectors but also present antigens to adaptive lymphocytes. Upon activation they upregulate CCR7, which drives their migration to the lymph nodes and then increase the expression of class I and II molecules as well as CD80 and CD86 inducing naïve $\alpha\beta$ -T cells²³⁴.

Despite the multiple physiological roles of $\gamma\delta$ -T cells and the great potential of this cell population as a therapeutic tool, some limitations need to be overcome, like the number and activation status of the infused cells or their appropriate tumor homing and tropism, that could be examined in pre-clinical *in vivo* human models.

II. HUMAN IMMUNE SYSTEM MICE

EARLY DEVELOPMENT OF RECIPIENT STRAINS

The interest of scientists in transplanting human cells into other living organisms came together with the discovery of cellular immunity itself in early 20th century. The nonphysiological conditions of in vitro systems and the poor recapitulation of some aspects of human hematopoiesis, disease susceptibility and pathophysiology in mouse models set the need for the development of better in vivo systems ²³⁵. However, transplantation of allogeneic or "non-self" cells or tissues into wild-type mice prompts a robust response that leads to graft rejection, only avoidable by the elimination or severe suppression of the murine innate and adaptive immune systems. Since the discovery of the nude athymic mutation (that provoke a T cell deficiency) in the 60s, chimerism levels and functionality of the engrafted human cells have been enhanced thanks to the improvement of the immunodeficiency in host strains ²³⁶. The first successful engraftments of human hematopoietic stem cells came in the late 80s with the triple deficient *beige/nude/xid* mice that on top of being athymic they have reduced NK cells and B cell functionality ²³⁷ and also the SCID (severe combined immunodeficiency) mice that due to the deficient activity of a DNA repair enzyme (Prkdc; protein kinase, DNA activated, catalytic peptide) they cannot execute the adaptive V(D)J recombination ²³⁸. The SCID mutation in CB17 mice permitted engraftment of PBMCs, fetal hematopoietic tissues and HSCs ²³⁹⁻²⁴¹. Despite that remarkable accomplishment, the presence of innate immune cells and the leakiness of host adaptive lymphocytes with aging limited considerably the extent of the human compartment ²⁴². Additionally, the deficient DNA repair machinery leads to an enhanced sensitivity to radiation, preventing the conditioning of the mice before transplantation. This could be overcome with the description of the mutation in the recombination activating genes (Rag) 1 and 2, although the intact NK cell activity still prevented a good HSC engraftment^{243,244}. A substantial improvement in the field came with the crossing of the SCID mutation into the NOD (non-obese diabetic) background (NODscid) further providing a certain innate immunodeficiency and dramatically reducing the adaptive leakiness evidenced by the low levels of serum immunoglobulins ²⁴⁵. Still, the short life-span due to the development of thymomas and the presence of residual innate cells limited their usage as a model. The development and homeostasis of both T and B-

lymphocytes and particularly NK cells greatly depend on cytokine signaling, mostly through the receptors that contain the common cytokine-receptor gamma chain (IL-2R). Its targeted mutation blocks therefore signaling through IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, and consequently leads to severe impairment of adaptive lymphocytes and total absence of NK cells ²⁴⁶⁻²⁴⁸. Several IL2ry-targeted mutations were then developed by different labs in various inbred strain backgrounds generating some of the models used nowadays ²⁴⁹⁻²⁵². Among them, NOD/.Cg-Prkdc^{scid} IL2r^{tm1wj}/Szj (NSG), NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}/Jic (NOG) and BALB/c $Rag2^{-/-}$ $Il2rg^{-/-}$ (BRG) have shown the best ability to support human engraftment They are similar in phenotype showing no mature T and B cells and severe defects in NK cells and other innate effectors and thus leading to a long-lasting development of a functional human immune system, improving by 6-fold the human cell engraftment compared to NODscid mice. Unlike the first two models, BRG mice present intact DNA damage repair machinery and are therefore resistant to radiation ²⁴⁵. The considerable advancement obtained with the IL-2R by the abolition of NK cell development was also achieved to some extent through the mutation of perform and beta2-microglobulin (B2m) genes generating the corresponding mice models ^{253,254}.



Figure 13: Improving homeostasis and function of human immune cells in HIS mice.

Among all the backgrounds used over the years the overall level of reconstitution is better in the NOD based models and this is due to an enhanced phagocyte tolerance towards human cells. The molecular basis underlying this advantage was revealed some years later by the genetic demonstration that the NOD Sirp- α showed enhanced binding to the human CD47 ligand ²⁵⁵. CD47 is ubiquotusly expressed in healthy human cells and its binding to SIRP- α (in phagocytes such as macrophages) releases an inhibitory signal that prevents their phagocytosis ²⁵⁶. The *Sirpa* gene is highly polymorphic and while in most mouse strains it does not interact functionally with the human CD47, the similarity of the *Sirpa* allele between the NOD strain and the human allows an operational signaling thus supporting human engraftment. Several methods have been used to induce tolerance based on this interaction: introducing the human *SIRPA* gene into the non-NOD genome using BAC-transgenesis or *knock-in* technology ²⁵⁷ or expressing the murine CD47 in the transplanted human cells (Figure 14) ²⁵⁸.



Figure 14: Approaches to improve xenograft tolerance in HIS mice on the basis of the CD47-SIRPα interaction.

Besides a murine deficient innate and adaptive immunity that prevents the graft rejection, an appropriate niche needs to be created to physically support the settlement of human cells. This can be achieved either by the pre-conditioning irradiation of the host or by the reduction of murine HSCs by eliminating key stemness genes (like *c-kit*) or replacing them by their human counterparts $^{259-261}$. Even after successful engraftment of HSCs, development and differentiation into functional mature immune cells requires the complex interplay of cell-mediated interactions and soluble factors like cytokines. Needless to say that in such a murine environment, those interactions are not always functional and only a few

murine cytokines cross-react with the human receptors. That is the reason for the poor reconstitution of the myeloid compartment and the innate lymphoid cells as well as the deficient maturation of the B cells (few memory and plasma cells) ^{262,263}. Additionally the lack of a human thymic niche prevents the selection and maturation of T cells and those that develop are selected in murine cortical epithelial cells that express murine MHC (H2) molecules ^{264,265}. This characteristic leads to a much higher B/T ratios in HIS mice than those observed in the human system (Figure 15).



Figure 15: White blood cell composition in human blood versus humanized mice (based on the models BRGS/NSG reconstituted with FL or CB derived CD34+ HSCs)

The search for the best model organism prompted the modification of the different parameters that could have an impact on the human cell composition and functionality thus generating a number of models that may adjust better to particular scientific questions. These modifications include the injection route that maybe intravenous (in tail or facial vein), intrahepatic, intrasplenic, intrafemoral, intracardiac or intraperitoneal. The route normally depends on the type of cell and the age of the mouse. Both newborn mice and adult mice are produced with similar overall human reconstitution but the latter generating less T cell reconstitution ^{250,263}. Newborn mice are normally injected intrahepatically or intracardically while adult mice receive the human cells intravenously. Regarding the source of human cells PBMCs are only used for specific purposes in short-term experiments while the majority of the research in the field now is performed with HSCs. These stem cells may derive from fetal liver, cord blood or PB/BM after mobilization with G-CSF, following that order in the engraftment efficacy ^{266,267}. Most of the humanizations are carried out after a niche conditioning that can be done by radiation or through drugs with the first reporting better results ²⁶⁸.

CURRENT HIS MOUSE MODELS

i. HU-PBL-SCID

Due to the difficult access to fetal samples, some researchers have opted for the use of peripheral blood mononuclear leukocytes as a source of human cells to inject in either BRG or NSG mice. The applicability of this model is limited since the T cells have been educated in the human thymus donor and can therefore become anergic after chronic activation or cause a xeno-GVHD reaction ^{269,270}. Consequently, primary immune responses are rare in this model and its applicability has been focused on the modeling of alloreactivity and the short-term study of infectious agents. Skin and islets graft rejections have been performed in this model but PBMC donor variability limits the recapitulation of the complete human graft rejection ²⁷¹.

ii. NSG AND BRGS

Although the genetic backgrounds of these two models are very different: NOD/.Cg-Prkdc^{scid} IL2r^{tm1wj}/Szj and BALB/c Rag2^{-/-} Il2rg^{-/-} NOD.Sirpa, they can be studied together because of their similar characteristics. Usually (including the work presented in this thesis) these two mice are engrafted with fetal liver or cord blood HSCs (CD34⁺ cells) through intrahepatic injection of newborn pups that have previously been sublethally irradiated. In this context, both NSG and BRGS mice showed a high and long-lasting human CD45⁺ compartment ^{249,258}, with B, T and myeloid cells developing in blood with an average ratio of 7:2:1. Some but infrequent NK cells are also present in peripheral organs with deficient functionality. B cells are detected in blood after the first month and several precursor stages are observed with a profile that resembles that in humans. The B-cell repertoire seems broad judging by the IgM responses found upon immunization with a range of antigens and some antigen-specific IgGs suggesting a proper class-switching ²⁷². The phenotypic T cell profile in the thymus of these mice suggest a normal differentiation pathway, however their functionality remains suboptimal. While EBV infection models have shown specific IFN-y producing CD8⁺ T cell protection from lymphoma development, other studies found that ex vivo stimulated T cells failed to respond in an antigen specific manner ²⁷³. NK cells are detectable in these mice from the fourth week post-reconstitution although in very low numbers unless they receive exogenous administration of cytokines such as the IL-15-IL-

15Rα complex or Flt3L ²⁷⁴ or endogenous secretion by transgenesis of human IL-2 ²⁷⁵ (Li et al., in press). Myeloid cells including monocytes, macrophages and DCs (conventional and plasmacitoid) are present in lymphoid organs (BM and spleen) and also in lung and liver and they proved to be functional in producing cytokines and activating T cells; nevertheless their limited numbers in absence of transgenic or exogenous cytokines is very low (less than 1% in spleen) ^{276,277}. The most dramatic difference in the composition of the hematopoietic compartment in human versus the HIS mice are proportion of granulocytes and erythrocytes (Figure 15). These cells account for less than 2 % (if found) and can only be enhanced through transgenesis of cytokines (SCF, GM-CSF, IL-3 and/or TPO) or hydrodynamic injection of plasmids ^{274,278}.

iii. **BLT** (BONE-MARROW, LIVER, THYMUS)

In an attempt to circumvent the problem of the T cell education in the late 80s a mouse model was generated by engraftment of human fetal lymph node and thymus under the renal capsule of NOD-scid mice followed by the intravenous injection (after irradiating preconditioning) of human CD34⁺ cells ^{240,279}. However, the confinement of the human cells to the organoid and not to other tissues of the mouse limited its utility. A further improvement of this approach came with the transplantation of fetal liver and thymus before the injection of CD34⁺ from the same liver into an immunodeficient mouse, NOD-scid, NSG or BRG (BLT mice) ²⁷⁹. In this model we can find the systemic high levels of adaptive lymphocytes, monocytes, macrophages and DCs. Moreover, the presence of a human thymus leads to a humanized selection of T cells thus mounting HLA-class-I and –II restricted responses and high levels of IgM, although recent studies show a deficient class-switch antibody production ^{279–281}. BLT mice have been infected with several hematotropic pathogens such as EBV, dengue or HIV-I generating humoral and cellular immune responses that in the case of HIV has contributed to the understanding of the viral latency, mucosal transmission, potential immunotherapetics and the *in vivo* reservoir ²⁸².

iv. MITRG AND MISTRG

The aforementioned limitations regarding the number and functionality of the myeloid compartment and NK cells due to the lack of cross-reactivity of mouse cytokines can be partially circumvented by the genetic humanization of the aforementioned mouse strains. Using this approach the laboratory of Richard Flavell created the MITRG and MISTRG that

contain the human M-CSF, IL-3, GM-CSF and TPO genes by knock-in technology and SIRPα by using a BAC transgene (in the case of MISTRG) ²⁸³. These models, especially the MISTRG, support the development of myeloid-monocytic cells as any other model and also show strong human innate immune responses to virus and bacteria infections. Furthermore, the increased numbers of monocytes and DCs lead to an enhanced trans-presentation of IL-15 resulting in a 10-fold increase of NK cells. However, these great results are counteracted by the early and high mortality of mice caused by a severe anemia due to poor mouse erythropoiesis.

Models addressing adaptive primary and secondary responses have been developed relying on the transgenic expression of human HLA-I and -II molecules in some of the strains aforementioned. These models show an increased number and function of HLA- restricted T cells with antigen-specific responsiveness following immunization or infection as well as a immunoglobulin class switching capacity thus responding efficiently to vaccination strategies ^{284–289}

HARNESSING IMMUNITY TO BATTLE TUMORS: LESSONS FROM HIS MICE

Therapies designed to induce or potentiate the immune response against tumors are an appealing strategy to control tumor growth and have been the object of intense research since their discovery in the 70s. Despite representing the most promising cancer treatment since the emergence of chemotherapy, several cases of side effects or disappointing clinical results have downshifted the development of new immunotherapies. The better understanding of the tumor heterogeneity, the mechanisms of the immune response and the interaction with the tumor microenvironment is a required step for the development of safe and effective therapies.

i. MODELING CANCER IN HIS MICE

Immunodeficient mice award great flexibility for the study of human tumor immunobiology with tumors arising from simpler tumor cell lines to the transplantation of human primary tumors or the *de novo* induction of hematological neoplasms that better reflect the complexity of the disease. Furthermore, as detailed earlier, the immune components can be generated *in vivo* from human HSCs or other progenitors and "supported or potentiated" later on, or infused once the tumor is established as a therapeutic approach.

A wide range of established tumor cell lines from different origins (brain, colon, breast, melanoma, ovarian, prostate, etc.) have been engrafted in immunocompromised mice and have greatly contributed to drug development and the pre-clinical assessment of potential therapies. However, the modification of the characteristics and acquisition of genetic aberrancies accompanying the repeated passaging impact the surface markers and the tumoregenicity of the heterogeneous cell populations that normally form the tumor ²⁹⁰. These limitations have set aside these models to studies addressing specific questions like the ability of a potential therapy to target certain molecule that has been overexpressed in the cell line. In recent years, the field has been therefore switching towards the engraftment of patientderived primary tumors (PDX, patient-derived xenografts) that keep the phenotypic and genetic complexity observed in the clinic. This include tumor stromal cells, such as tumorassociated lymphocytes and fibroblasts, that contribute greatly to tumor growth and metastasis and therefore to the therapeutic response. PDX mice that have been reconstituted with human HSC or PBMCs represent the latest step in immuno-oncology modeling ²⁹¹. These models can engraft the PDX as efficiently as the non-humanized mice, they respond to standard chemotherapeutic drugs similarly to patients and they have proved to be responsive to newly derived immune-modulators. One of the better-characterized PDX models is the AML that has contributed to the identification of leukemia stem cells (LSC) by transplanting different stem-like cell fractions and analyzing the leukemia-initiating activity of each in SCID mice ²⁹²⁻²⁹⁵. The discovery of the concept of cancer stem cell (CSC) has been a breakthrough in cancer biology due to the clinical implications for the long-term disease-free survival. CSC presence has been identified in numerous malignancies through transfer into immunocompromised mice and, interestingly, markers associated to CSCs have been correlated to the tumorigenic potential ^{296,297}. Recent improvement in HIS mouse models by the transgenic expression of certain factors, like the NSG-SGM3 expressing SCF, GM-SCF and IL-3 could be of benefit for the engraftment of myeloid derived malignancies and the study of tumor infiltrating myeloid cells ²⁷⁸. The demonstration that HIS mouse models reproduce the heterogeneity and behavior of human tumors create great expectation on the better phenotyping of these tumor-initiating cells and the identification of drugs targeting this population ²⁹⁸.

One of the advantages of modeling cancer in humanized mice is that we can study the systemic environment and the contribution of nearly all the immune cells to the pathogenesis. In this regard, a NSG model of treatment-refractory B-cell leukemia revealed that infiltration

of leukemia cells into the bone marrow rewires the tumor microenvironment to inhibit engulfment of antibody-targeted tumor cells. This resistance could be overcome by combination regimens involving therapeutic antibodies and chemotherapy that lead to macrophage infiltration and phagocytic activity in the bone marrow improving the efficacy of targeted therapeutics²⁹⁹.

ii. TARGETING NATURAL KILLER CELLS IN HIS MICE

To date most immuno-modulatory strategies have focused on agents or cell therapies targeting T cell immunity but innate immune cells have been less exploited. NK cells exert an immediate cytotoxicity when encountering a malignant cell and they do so without a specific antigen priming but instead, by the integrated signal of an array of activating and inhibitory receptors. Among the first group the C-type lectin-like receptors CD94/NKG2C and NKG2D and the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 as well as the Iglike receptor DNAM-1 (CD266) mediate NK cell activation when they recognize tumor cells. On the other hand, polymorphic inhibitory killer cell immunoglobulin-like receptors (KIRs) with their cognate human-leukocyte-antigen (HLA) ligands as well as CD94/NKG2A with the non-classical class I molecule HLA-E as ligand provide inhibitory signaling. In addition to the contact mediated regulation of the activity, NK cells also respond to cytokines like IL-2, IL-12, IL-15, IL-18 and IL-21, as well as toll-like receptor ligands that shape their differentiation, proliferation and activation status ³⁰⁰. Cytotoxicity activity is triggered through activation of the low-affinity activating receptor FcyRIIIa (CD16) that binds the Fc portion of immunoglobulin G1. NK cells kill virus infected and tumor cells using a cargo of perforin and granzymes contained in cytotoxic granules and less efficiently by a mechanism dependent on FAS ligand, TNF or TNF-related apoptosis-inducing ligand (TRAIL)³⁰¹.

Given that NK cells in HSC-derived humanized mice express all the afore-mentioned receptors and respond similarly to the same cytokines, these *in vivo* models represent a powerful platform to explore the pivotal role of NK cells in cancer immunosurveillance ³⁰²⁻³⁰⁴(BRGSF?). Additionally, stromal components such as inhibiting factors (TGF- β , IL-10, prostaglandin E2...) or immunosuppressive cells (T_{regs}, MDSCs) that influence the anti-tumor activity of NK cells are, as well, present in HIS mice enabling the evaluation of therapeutic strategies targeting the suppression of NK cells ³⁰⁵.

Although chemotherapy is still the core of the current clinical anti-cancer treatments, immuno-modulators have now regained expectations after the revolutionary discovery of the

CTLA-4 and PD-1 checkpoint inhibitors targeting T-cell activation ³⁰⁶. Humanized mice have proven to recapitulate the therapeutic effect of those antibodies as well as the side effects and have began to provide insights about the mechanism behind and possible strategies to improve them ^{307–309}. Human NK cells also express these checkpoint molecules but their targeting and contribution to the results of the treatment has not been assessed. Mouse studies on a glioma model treated with activated NK cells pre-incubated with an anti-PD-1 blocking antibody showed an enhancement of the survival suggesting a role that must be explored in a human system ³¹⁰.

iii. ADOPTIVE NK CELL TRANSFER

Additionally to HSC-derived HIS models in which NK cells develop *in vivo* and in the context of other immune cells, the potential of these innate effectors has been studied by the adoptive transfer of *ex vivo* expanded and/or activated NK cells in immunodeficient mice. Mice treated with adoptively transferred human NK cells show NK-mediated rejection of the engrafted human tumor and further administration of cytokines, such as IL-2 and IL-15 greatly improve the NK cell pool and their cytotoxic activity against transformed cell. These observations initially made in mice laid the foundation for the **autologous NK cell infusion** therapies started in the 80s for metastatic cancers ³¹¹. Pre-clinical assessment of cytokine regimes in other cancer models, such as the low-dose IL-2 in the spontaneous EBV-associated B cell lymphoma in PBL-SCID mice, demonstrated reduction of the tumor load and survival prolongation ³¹², and preceded a number of clinical trials for both hematological and solid tumors (91,92,96–98).

The discovery that inhibitory KIRs binding to MHC-I mediate inhibition of NK cell activation opened a new path on NK cell immunotherapies. NOD/SCID cancer models served as a platform to confirm the higher efficacy of **alloreactive NK cells** for the treatment of leukemia. Contrarily to T cells, NK cell do not provoke graft-versus-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT) contexts but, instead, protect the patient against it and eliminate leukemia relapse and graft rejection ³¹⁸. Later on, safety and efficacy of alloreactive NK cell infusion was confirmed in the clinic by Miller and colleagues in non HSCT settings with patients suffering from metastatic melanoma, renal cell carcinoma, Hodgkin's lymphoma and refractory AML ³¹⁹. For many years allogeneic NK cell infusions have been tested in the clinic with positive results and rare cases of mild toxicity ³²⁰. Nevertheless, a recent pediatric clinical study has reported some patients suffering from acute

GVHD after infusion of *ex vivo* expanded donor NK cells in HLA-matched HSCT ³²¹, rising the necessity to perform more robust pre-clinical testing in humanized models. One strategy to do so was illustrated in a recent study performed in NOD/SCID mice, in which an alloreactive NK cell subpopulation expressing KIR2DS2 but lacking inhibitory KIR-HLA mismatch had dominant functional activation advantage to kill patient-derived glioblastoma cells ³²². The regulation of the activity on infused NK cells has been classically based on HLA-KIR matching, however other inhibitory receptors are implicated on the inhibition of NK cell cytotoxicity. A recent study in HSC-NSG mice has shown that anti-NKG2A antibodies can stimulate human NK cell killing in AML and ALL models bypassing the need to search for NK cell alloreactive donors ³²³. *In vitro* experiments have also pointed to an increased NK cell-mediated lysis of lymphoma and myeloma cells with allogeneic NK cell infusion in combination with monoclonal antibodies blocking inhibitory KIRs but this effect need to be confirmed *in vivo* ^{324,325}.

Another strategy to increase NK cell activity without aggravating the side-effects is the expression of chimeric antigen receptors (CARs) directed against tumor antigens. Preclinical evaluation of CD20 targeting primary NK cell infusion in humanized mice has led to a clinical trial on B-lineage acute lymphoblastic leukemia currently undergoing ³²⁶. Other pre-clinical trials using CAR-engineered primary human NK cells are now being performed in lymphoma, leukemia, carcinomas and neuroblastoma mouse models.

NK cells are often infused in combination with immunomodulators that boost their anti-tumor effects or regulate their activity. CD16 receptor is in the origin of most of those modulators since it mediates antibody-dependent cellular cytotoxicity (ADCC) when it recognizes an antibody on a target cell, leading to target cell lysis. This mechanism has been exploited by using monoclonal antibodies targeted to tumor antigens thus stimulating the endogenous or adoptive NK cells. Evidences of NK cell-mediated ADCC and mild to moderate toxicity was observed in preclinical models and then confirmed for some cases in the clinical setting. Malignancies such as non-Hodgkin lymphoma with rituximab (anti-CD20), metastatic breast cancer with trastuzumab (anti-HER2) or metastatic colorectal and squamous cell carcinoma of the head and neck have been treated with monoclonal antibodies together with NK cell infusions or in combination regimes extending the disease-free survival and overall survival of thousands of patients ³²⁷⁻³²⁹.

As mentioned before, CSCs are emerging as necessary targets to achieve cancer cures since current treatments eliminate the bulk of the tumor cells but rare resistant CSCs persist
and lead to later tumor relapse ³³⁰. The upregulation of stress-induced antigens together with the ability of NK cells to target non-proliferating cells suggest that NK cells could effectively eliminate CSCs. Indeed, recent studies in tumor-bearing NSG mice demonstrated the capacity of activated transferred NK cells to reduce intratumoral CSCs and tumor burden ^{331–333}.

iv. NOVEL NK CELL SOURCES

Two of the parameters to consider when evaluating the safety of **NK cell products** in clinical applications are the cell source and the culture conditions before the infusion. GM-CSF mobilized PBMCs, bone marrow or umbilical cord blood are the main sources of NK cells. With GM-CSF effects on NK function still to determine and BM being logistically difficult to obtain, UCB derived NK cells have been revealed as the best source of human material. Researchers are working on improving the expansion yield and purity as well as to enhance the activity of UCB derived NK cells before infusion in the patients. NSG mice demonstrated the capacity of these cells to migrate to BM, spleen and liver and the inhibition of leukemia growth and prolongation of mice survival when combined with low-dose IL-15 ³³⁴. This pre-clinical results prompted a phase I clinical study in elderly AML patients that confirmed the safety and capacity of these cells to migrate and repopulate BM even in the absence of cytokine administration ³³⁵. This NK cell product aims at overcoming the major limitation of NK cell therapies in solid tumors, the delivery of high enough numbers of activated NK cells to the tumor site, and it is now under pre-clinical evaluation in the context of cervical and colorectal carcinomas ³³⁶.

Recently emerged alternative sources include the embryonic stem cell (hESC) or induced pluripotent stem cell (iPS) derived NK cells, which are still under experimental development. Efficient generation of NK cells from hESC and iPS cells has been achieved showing *in vitro* functional cytolytic activity against tumor cells, IFNγ production and expression of functional receptors ³³⁷. Very few reports are available regarding the *in vivo* activity of these products, with the more encouraging being in a NOD/SCID mouse model in which hESC-derived NK cells efficiently cleared a leukemia cell line tumor ³³⁸. Current efforts are being made to optimizing the generation and efficacy of the stromal-free production but there is still a long way to go before reaching the clinic to prove irreversibility and safety of hPSC therapy in pre-clinical humanized models.

Finally, there is great prospect in NK cell lines as a potentially unlimited "pure" NK cell source. From the handful of available clonal NK cell lines NK-92 has shown the highest

and more consistent cytotoxicity due to the combination of activating receptors it expresses and the absence of inhibitory KIRs ³³⁹. AML, myeloma and melanoma are some of the numerous malignancies that have been partially eliminated from SCID mice after infusion of NK-92 ³⁴⁰⁻³⁴². Clinical trials have further confirmed the safety and efficacy of this cell line in both solid and hemotologic malignancies ^{343,344}. One further advantage of NK-92 is the ease of transfection with non-viral vectors allowing them to express IL-2 (required for their proliferation) thus representing a powerful "off-the-shelf" cell therapeutic ³⁴⁵. Additionally and inspired by the remarkable responses obtained by CAR-T cells and the early results in primary NK cells, NK-92 can be very easily transfected with a gene that expresses a tumor-CAR ³⁴⁶. The first pre-clinical tests in NSG mice have shed very optimistic results in leukemia models after CD19- or CD20-specific NK-92 infusions as well as in patient-derived glioblastoma with EGFR-specific NK-92 ^{347,348}.

The latest of the NK cell therapeutic strategies was developed by Vallera and colleagues with the bi-or tri-specific killer cell engagers, BiKEs and TriKEs, that are small molecules containing two or three single chain variable fragments (scFv) from antibodies of different specificity ³⁴⁹. These are generated to bind CD16 on NK cells and one or two tumor antigens such as CD19 and CD20 (B cell non Hodgkin's lymphoma) ³⁵⁰, CD33 or CD33 and CD123 (AML) ³⁵¹, CD30 (Hodgkin's lymphoma) ³⁵², EGFR or EpCAM (EGFR/EpCAM overexpressing carcinomas) ^{353,354}, and many others. The initial pre-clinical evaluation in humanized mice proved very promising translational potential with results exceeding those of monoclonal antibodies, like in the case of CD16-CD19-CD20 TriKE versus rituximab, and also prove efficient for overpassing HLA-mediated inhibition in refractory AML blasts.

IL-15 is the master cytokine necessary for NK cell differentiation and survival and it is currently used in clinical trials alone or as an adjuvant for certain types of metastatic solid tumors to promote *in vivo* cell expansion and NK cell function ^{302,355}. Taking advantage of this, novel TriKE structures have been developed that use a human IL-15 as a modified cross-linker between the anti-CD16 and the anti-tumor antigen in order to promote *in vivo* NK cell proliferation. Assessment of the activity of a CD33 specific TriKE in an AML NSG model of NK cell adoptive transfer has shown *in vivo* persistence, high cytotoxic activity and no toxicity to the construct ³⁵⁶. Clinical development is currently under progress and will probably obtain FDA approval in the upcoming months to be tested in patients.

IMPROVING THE NK CELL COMPARTMENT IN HIS MICE

Given the central role of NK cells in immune responses in infection, malignancy and inflammation and the great therapeutic potential they hold, it is necessary to optimize the available models for understanding their biology and pre-clinically evaluate new therapies.

In previous sections we discussed about two types of HIS mice for the study of NK cell biology, those in which the human immune cells develop *in vivo* from injected hematopoietic precursors and a second category that adoptively receive mature NK cells freshly isolated or derived from an *ex vivo* expansion or activation process, a cell line or an ES or iPS cell. The later have fewer requirements in terms of niche, cell-cell interactions and soluble growth factors that are needed for NK cell development, and instead require cytokines for their survival and homeostatic proliferation. These cytokines are grouped by the use of the common cytokine receptor γ chain: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Particularly, IL-15 is the responsible for NK cell maintenance and homeostatic proliferation through IL-15R α presentation ^{357,358} while IL-2 effect *in vivo* is oriented to the activation and induction of cytotoxicity through the regulation of the peripheral NK subsets. These humanized mice serve as platforms to understand the mechanisms underlying NK survival and function and provide pre-clinical information for the design of new therapeutics. Furthermore, they give valuable information about the cell migration capacity and synergistic effects with other cell types or immunomodulators.

As mentioned earlier immunodeficient mice carrying *IL-2rg* mutations like NSG or NOG (both NOD/SCID/Il2rg) and BRG (Balb/c *Rag/Il2rg*)) support de novo multi-lineage development of human immune cells, including low levels of NK cells. In the BRG model an improvement of human reconstitution, including NK cells, has been observed when expressing SIRP α^{NOD} as indicated earlier either by transgenesis or by generation of the congenic strain ^{257,258}. These NK cells expressed CD56 and NKp46 as well as some level of CD16 and were able to degranulate moderately after stimulation with a cancer cell line. However in both BRGS and NSG mice NK cell displayed defects in maturation, functionality and heterogeneity in comparison with the human counterparts due to a deficient cytokine signaling ³⁵⁹. Based on their cytokine requirements IL-15 has been exogenously administered either alone or as a complex with IL-15R α resulting in an extensive NK cell proliferation and accumulation of CD16⁺KIR⁺ NK cells. Also, NK cell differentiation progressed from CD56⁺ to CD56^{low}CD16⁺, and finally to CD56^{low}CD16⁺KIR⁺ mimicking the human model ³⁰². On the other hand, the constitutive high expression of the high-affinity heterotrimeric IL-2 receptor

complex in CD56^{bright}CD16^{+/-} NK-cell subset and the effect of IL-2 in NK expansion and activation prompted the development of and IL-2 transgenic NOG mouse strain ³⁶⁰. When IL-2^{Tg}NOG mice were engrafted with human HSC, CD56⁺ massively developed with a highly active phenotype including IFNγ production and cytotoxicity against tumor cells. Treatment with a therapeutic humanized anti-CCR4 Ab (mogamulizumab) suppressed the growth of a CCR4⁺ lymphoma, suggesting that the human NK cells in the mice exerted active Ab-dependent cellular cytotoxicity *in vivo*. These cells expressed various NK receptors, including NKp30, NKp44, NKp46, NKG2D, and CD94, as well as a diverse set of killer cell Ig-like receptor molecules at levels comparable to normal human NK cells from the peripheral blood ³⁶¹. Nevertheless, there are several limitations in this model due to the supra-physiological levels of IL-2 and therefore the high activation status of the NK cells.

It is well known that NK homeostasis and function is regulated by the interaction with other immune cells, particularly macrophages, dendritic cells and T cells. In addition, soluble factors released by those cells, like NKG2D ligands, IL-2, IL-12 or IL-15, signal on NK cells leading to proliferation and activation. Based on these crosstalk events, others and we have developed humanized mice that through the enhancement of the myeloid compartment, NK cell development results improved. As happens for other lineages, human myelopoiesis is driven by soluble factors normally present in the BM niche and periphery that in HIS mice are from mouse origin. Some of those mouse cytokines cross-react to some extent with the human cells but others, the species-specific cytokines, do it very poorly. In order to circumvent this deficiency human cytokines have been administered to HIS mice either as recombinant proteins ³⁰², by cytokine-encoding plasmids ³⁶² or by insertion of the cytokines either as transgenes in the mouse genome or by knocking in the human gene to replace the mouse counterpart ^{363,364}. As mentioned before, transgenic models provoke supraphysiological levels of the cytokine in the periphery and in the case of pro-myeloid factors, such as TPO, IL-3, GM-CSF or M-CSF, also lead to the exacerbated mobilization and HSC exhaustion limiting the utility of the system. The knock in approach, like the MITRG and MISTRG, allow a more physiological expression but adaptive cellular and humoral responses are very poor and the deficient mouse erythropoiesis leads to severe anemia that limits the lifetime of the animals. Subsequently to the myeloid enhancement, both these models showed an increased NK cell compartment as for the number of cells and NK cell functionality, including higher expression of KIR, CD94 and CD161 receptors ²⁸³. The transpresentation of II-15 occurs mainly through the IL-15R α expressed by dendritic cells so efforts have been

made to increase specifically this cell population in order to increase the NK pool avoiding the overdevelopment of other myeloid subsets. In our laboratory, Flt3-deficient BRG mice (BRGF) were created and after reconstitution with human HSC, human Flt3L was administered to the mice. The result was an increase of all the DC subsets and the promotion of NK cell hematopoiesis, with enhanced CD94, CD16 and KIR receptor expression. The combination of this system with the expression of the Sirp^{NOD} protein in the BRGSF model has led to further augmentation of NK cell numbers and also an enhanced functional competency as for degranulation capacity and cytokine production activity (unpublished data). This HIS model provide a unique platform to study NK cell development, crosstalk mechanisms with other immune cells and the pre-clinical assessment of new immunotherapies targeting innate cells.

The combination of the protocols detailed in the previous sections for modeling human cancer or infection with the strategies abovementioned to boost the NK cells in HIS mice will rise the potential to understand how NK cell interact with malignant or infected cells. Moreover, HSC-HIS mice offer the possibility to study the tissue specific interactions, the reservoirs, the migration patterns and the crosstalks within the immune compartment that may be important to develop combinatorial therapies that avoid metastasis, tumor relapse and "relocation" of the virus.

SPECIFIC AIMS OF THE THESIS

The overall objective of my thesis studies was to improve the available human immune system mouse models for the study of human innate effectors, their biology and their therapeutic potential.

- I. Develop a HIS mouse model capable of recapitulating the phenotype and functions of human innate lymphoid cells.
- II. Interrogate the developmental stages that give rise to human innate lymphoid cells *in vivo* in HIS mice.
- III. Develop and evaluate novel innate immune cell-based cancer immunotherapeutic approaches in HIS mice.

RESULTS

PAPER I: A FUNCTIONAL DC CROSSTALK PROMOTES HUMAN ILC HOMEOSTASIS IN VIVO

Genetic engineering has allowed the tracking of the ontogeny of mouse ILCs that is currently quite well known. However, the human ILC development has been studied using different cell precursor origins and culture conditions thus creating discrepancy among the groups. *In vivo* models of human ILC biology are needed to reconcile those models, understand the signals and dissect the stages of human ILC development and therefore ILC diversity and function. Given their rapid response to infection, stress or injury and the aforementioned involvement in many pathologies, they may be ideal targets to modulate the response in inflammatory conditions, immunotherapy or vaccination.

ILC differentiation involves the interaction of precursor cells with stroma and myeloid cells and the signaling through soluble factors produced by those. However, human myeloid cells are not efficiently reconstituted in HIS mice due to the lack of human cytokines and the competition for the environmental niche in mouse tissues. One of the cytokines necessary for myeloid development is the FMS-related tyrosine kinase 3 ligand (Flt3L).

In this study we generated a <u>BALB/c</u> <u>Rag2⁻¹-Il2rg⁻¹-Sirpa^{NOD}<u>Flk2</u>⁻¹ (BRGSF) HIS mouse model in which Flt3L-mediated enhancement of human DC numbers resulted in improved human ILC cell homeostasis and function. We provide evidence for a functional crosstalk between human DC and human ILC subsets that occurs in across lymphoid and non-lymphoid (mucosal) tissues. Based on that, BRGSF HIS mice open new possibilities for the study of ILC development and associated pathologies as well as the assessment of new immunotherapeutic strategies.</u>

A functional DC crosstalk promotes human ILC homeostasis in humanized mice

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Key points:

- A novel humanized mouse model to study human ILC biology

- Human DC crosstalk with innate lymphoid cells in vivo

Abstract

Humanized mice harboring human hematopoietic systems offer a valuable small animal model to assess human immune responses to infection, inflammation and cancer. Human Immune System (HIS) mice develop a broad repertoire of antigen receptor bearing B and T cells that can participate in adaptive immune responses after immunization. In contrast, analysis of innate immune components, including innate lymphoid cells (ILC) and NK cells, is limited in current HIS mouse models partly due to the poor development of these rare lymphoid subsets. Here we show that novel DC-boosted HIS mice harbor abundant NK cells and tissue-resident ILC subsets in lymphoid and non-lymphoid mucosal sites. We find that human NK cells and ILCs are phenotypically and functionally mature, and provide evidence that human DC activation in HIS mice can 'crosstalk' to human NK cells and ILCs. This novel HIS mouse model should provide the opportunity to study the immunobiology of human NK cell and ILC subsets *in vivo* in response to various environmental challenges.

(162 words)

Introduction

Transplantation of human hematopoietic stem cells into immunodeficient mice has been employed for the last two decades to generate <u>H</u>uman Immune System (HIS) mouse models. HIS mice provide a translational bridge connecting fundamental studies to clinical application, and have contributed to the better understanding of human immunity and disease pathogenesis^{1,2}. Nevertheless, complete reconstitution of all human hematopoietic lineages and homeostatic long-term maintenance of all human immune cell types has not yet been achieved in these models³. Much progress has been made to improve overall human lymphoid and myeloid cell engraftment, and to enhance the development of HLA-restricted T and B cell responses. In contrast, the *in vivo* development of human innate lymphoid effectors, including innate lymphoid cells (ILCs), NKT, MAIT and/or $\gamma\delta$ T cells, in HIS mice remains suboptimal.

ILC comprise a recently identified family of immune effector cells that share many functional characteristics with CD4⁺ T helper ($T_{\rm H}$) and CD8⁺ T cytotoxic lymphocytes (CTL). As such, ILCs have been proposed as innate versions of $T_{\rm H}$ and CTL. ILCs derive from committed lymphoid precursors present in the fetal liver and adult bone marrow⁴. ILCs are categorized in three main groups according to their transcription factor signatures and their cytokine secretion patterns⁵. Group 1 ILC (ILC1) include T-BET⁺ cells that produce high levels of interferon- γ (IFN- γ). Amongst ILC1, two subgroups are appreciated that differ in expression of EOMES. Natural killer (NK) cells are EOMES⁺ and represent an extensively studied ILC subset with cytotoxic activity against virus-infected and tumor cells^{6,7}. A second ILC1 subset does not express EOMES and lacks cytotoxic granules but expresses the IL-7 receptor (CD127). These 'non-cytotoxic' ILC1 are found associated with epithelium in the liver, lung and intestine⁸. Group 2 ILC (ILC2) express the transcription factor GATA3 and produce type 2 cytokines, especially IL-5 and IL-13. ILC2 surface markers include CD127,

CRTh2, CD161 and CD25 (IL-2R α), and these systemically distributed cells have an important roles during infections (by viruses, parasites), in atopic conditions (allergy, airway hyper-responsiveness) and during tissue repair^{9–11}. Group 3 ILC (ILC3) express the orphan nuclear receptor ROR γ t and produce IL-17A and IL-22 after stimulation. ILC3 play important roles in maintaining barrier surfaces (especially mucosal sites) and for protection against infections by fungi and extracellular bacteria. Interestingly, ILC3 are increased in several inflammatory conditions (IBD, psoriasis) suggesting that this subset may provoke disease^{12,13}.

In principle, HIS mouse models can provide an opportunity to better understand the development, differentiation and function of human ILCs *in vivo*. Along these lines, several reports have described human NK cell generation in HIS mice^{14–17} and have underscored the importance of species-specific cytokines and macrophage tolerance for the development and function of these innate effectors¹⁸. In contrast, reports on other human ILC subsets in humanized mice are scarce and only in the context of infection or inflammation^{19,20}. Several ILC subsets inhabit mucosal tissues, and the reconstitution of these sites in most humanized mouse models appears rather limited (reviewed in ²¹). This may result from poor homing or maintenance of human hematopoietic cells due to incompatibilities in adhesion molecules, chemokine/chemokine receptors and/or growth and survival factors. A comprehensive analysis of human ILCs in HIS mice could provide new insights into how, when and where these cells develop.

Previous studies correlated FMS-related tyrosine kinase 3 ligand (Flt3L)-mediated expansion of mouse dendritic cells with a higher ILC cell survival and proliferation^{22–25}. We recently reported a humanized mouse model in which Flt3L-mediated enhancement of human DC numbers resulted in improved human NK cell homeostasis²⁶. In this report, we generate and characterize a novel <u>BALB/c</u> <u>Rag2^{-/-}Il2rg^{-/-}Sirpa^{NOD}Flk2^{-/-}</u> (BRGSF) HIS mouse model

with enhanced human ILC homeostasis and function. We provide evidence for a functional crosstalk between human DC and human ILC subsets that occurs in across lymphoid and non-lymphoid (mucosal) tissues. BRGSF-based HIS mice should provide the means to interrogate human ILC function in the context of infection and inflammation.

Methods

Mouse strains and generation of HIS mice

<u>BALB/c</u> <u>Rag2^{-/-}112rg^{-/-}Sirpa^{NOD}<u>F</u>lk2^{-/-} (BRGSF) mice were generated by extensive backcrossing of <u>BALB/c</u> <u>Rag2^{-/-}112rg^{-/-}F</u>lk2^{-/-} (BRGF;²⁶) mice to the <u>BALB/c</u> <u>Rag2^{-/-}112rg^{-/-}</u> <u>Sirpa^{NOD}</u> strain (BRGS;¹⁴). <u>BALB/c</u> <u>Rag2^{-/-}112rg^{-/-}Sirpa^{NOD}<u>F</u>lk2^{+/-} mice were subsequently intercrossed to create the BRGSF strain. HIS mice were generated as previously described^{14,27}. Briefly, fetal liver CD34⁺ cells were isolated using human CD34 microbead kit (Miltenyi Biotec) and subsequently phenotyped for CD38 expression by flow cytometry. Newborn (3–5 day old) received sublethal irradiation (3 Gy) and were injected intrahepatically with the equivalent of 2 × 10⁵ CD34⁺CD38⁻ human fetal liver cells. All manipulations of HIS mice were performed under laminar flow conditions. Experiments were approved by the ethical committee at the Institut Pasteur (Reference # 2007–006) and validated by the French Ministry of Education and Research (Reference # 02162.02).</u></u>

In vivo Flt3L treatment

HIS mice were injected intraperitoneally three times per week for 2 weeks with 5 μ g hFlt3L-Fc (BioXcell), commencing at 6-7 weeks after reconstitution. Control mice were injected with the same volume of PBS. HIS mice were analyzed 2-5 days after the last hFlt3L injection.

Hydrodynamic injection in HIS mice

Full-length human IL-2, IL-7, IL-25 and IL-33 cDNA clones were cloned in the mammalian cell expression vector pCMV-6-XL4 (Origene) and plasmids purified using endotoxin-free Plasmid-Maxi kit (Qiagen). Hydrodynamic injection in HIS mice was

performed as previously described²⁸. Briefly, 8-9 weeks old HIS mice were weighed and injected i.v. (tail vein) with cytokine encoding plasmids in 1.8 ml PBS (for 20g body weight) within 7 seconds using 27-gauge needles.

Cell preparation

HIS mice were perfused with PBS prior to tissue preparation. Spleen tissue was minced and erythrocytes were lysed using (BD Bioscience). Percoll density gradient centrifugation (GE Healthcare Life Sciences) was used for liver lymphocyte preparation. Lungs were dissociated using 1mg/ml collagenase D (Roche) for 45 min followed by percoll density gradient centrifugation.

Intestines were perfused with cold RPMI 1640 medium to remove feces, cut open longitudinally and washed. Tissue was cut into 1-cm pieces and agitated in pre-warmed medium containing 10 mM EDTA (Sigma-Aldrich) at 37°C for 30 min to remove epithelial cells. Remaining tissue was collected, minced and digested for 60 min in medium containing 5% FCS and Liberase (1 mg/ml; Roche). Lamina propria lymphocytes were enriched by percoll gradient centrifugation, filtered and washed. All cell preparation steps were performed using RPMI 1640 Glutamax (Life Technologies) 100 U/mL penicillin, 100 μ g/mL streptomycin (Invitrogen) unless otherwise stated.

Flow cytometry

Cells were incubated for 15 min with cold PBS (Life Technologies) containing 3% FCS, human and mouse FcR block (hIgG and 2.4G2) and a viability dye (eFluor 506; eBioscience #65-0866-14). After a washing step, cells were stained with fluorochrome-bound antibodies. To detect intracellular transcription factors and cytokines, cells were subsequently fixed and permeabilized using manufacturer's protocols (eBioscience; BD). Antibodies used are listed in Supplemental Table 1. Samples were acquired using an LSR FORTESSA (BD) and data were analyzed using FlowJo software (TreeStar; version 9.8.5 & 10.0.8).

In vitro stimulation assays

For *ex vivo* stimulation assays, human ILCs were enriched by depletion of cells expressing mCD45, hCD3, hCD5 and hCD19 using magnetic cell separation (Miltenyi Biotec) according to manufacturer's procedures. ILCs were plated in 96-cell plates at a density of 4×10^4 NK cells (hCD45⁺CD3⁻NKp46⁺) or 10^4 ILCs (hCD45⁺Lin⁻CD7⁺CD127⁺) per well. Cells were stimulated using various cytokines (including IL-15 (Peprotech), IL-12, IL-18, IL-23, IL-1b per well. Cells were stimulated using various cytokines (including in the presence of Golgi Plug (BD) for 4 h. In some cases, stimulation was performed with PMA (10 ng/ml; Sigma-Aldrich) plus ionomycin (1 μ g/ml; Sigma-Aldrich) in the presence of Golgi Plug (BD) for 3 h. For degranulation assays anti-CD107a antibody and monensin (1 μ g/ml; Sigma-Aldrich) was added to the media 1h after the beginning of the stimulation (1ul/well). In all cases, a non-stimulated control containing only medium was included.

In vivo stimulation assays

For NK stimulation, reconstituted HIS mice were injected ip with 50 µg polyI:C (InvivoGen) in PBS and analyzed 14 h later. For ILC stimulation, a cocktail of flagellin, R848 (Resiquimod) and bacterial lipopolysaccharide (LPS-EB ultrapure) (all from InvivoGen, 5 ug each) was injected ip and analyzed 6 h later.

Statistical analysis

GraphPad Prism version 6 (GraphPad Software) was used to perform statistical analysis. Statistical significance was evaluated by two-tailed unpaired Student t tests. The obtained \underline{p} values were considered significant when $\underline{p} < 0.05$.

Results

Human DC development in Flt3L-treated BRGSF-based HIS mice

We have previously described BALB/c Rag2^{-/-}Il2rg^{-/-}Flk2^{-/-} (BRGF) recipients that offer an approach to enhance human DC homeostasis in HIS mice²⁶ and characterized the BALB/c Rag2^{-/-}Il2rg^{-/-}Sirpa^{NOD} strain (BRGS) with enhanced human hematopoietic engraftment due to improved macrophage tolerance of human cells¹⁴. We combined these two models to create the <u>B</u>ALB/c <u>Rag2^{-/-}Il2rg^{-/-}Sirpa^{NOD}Flk2^{-/-}</u> (BRGSF) strain and analyzed HIS mice after intrahepatic transfer of human CD34⁺ HSC into newborn BRGSF recipients. BRGSF-based HIS mice showed robust reconstitution with up to 75% circulating human CD45⁺ cells (Figure S1A), including CD3⁺ T cells (5-30%), CD19⁺ B cells (60-85%), NK cells (2-6%) and conventional DCs (cDCs) (1-4%) (Figure S1B). Consistent with previous studies^{26,29-32}, we found that exogenous Flt3L treatment could significantly boost human myeloid cell development in BRGSF HIS mice (Figure 1). We examined four human CD3⁻CD19⁻NKp46⁻ HLA-DR⁺ myeloid subpopulations: CD14⁺ monocytes, CD123⁺ plasmacytoid DCs (pDCs), CD141/BDCA-1⁺ cDCs and CD1c/BDCA3⁺ cDCs. All four subsets were detected in bone marrow (BM) (Figure 1A) and spleen (Figure 1C) and to a lesser extent in lung and liver (data not shown) of BRGSF HIS mice as minor populations (1-5%) of total CD45⁺ cells. Flt3L treatment resulted in a 30- to 85-fold increase in absolute numbers of these myeloid subsets in the BM and a 3-fold increase in absolute numbers of pDCs and CD141⁺ DCs in the spleen (Figures 1B, D). Exogenous Flt3L had no effect on the Flt3-deficient mouse myeloid cells (data not shown) thereby allowing a selective boost of human myeloid cells in this context. These results confirm previous studies using BRGF recipients²⁶, but further demonstrate enhanced development of CD14⁺ monocytes in BRGSF HIS mice.

We next analyzed hematopoietic and myeloid precursor populations in the BM of reconstituted BRGSF HIS mice. Total numbers of CD34⁺ HSC was significantly higher in Flt3L-treated mice suggesting enhanced homeostasis (Figure S2A). This is in line with previous in vitro and in vivo studies demonstrating that HSCs express Flt3 and that signaling through this receptor induces proliferation of quiescent bone marrow HSCs and positively survival^{33,34}. impacts cell Human DC and monocyte restricted precursors (Lin⁻CD34⁻CD117⁺CD135⁺CD116⁺CD45RA⁺ cells) have been shown to have a BM origin prior to entry into the circulation^{35,36}. This population was detected in the BM of BRGSF mice (Figure S2B) and its frequency and total numbers were increased following Flt3L treatment (Figure S2C). These data suggest that the increased frequency and absolute numbers of myeloid cells in Flt3L-treated BRGSF HIS mice may result from an increase in the CD34⁺ HSC pool and their downstream DC/monocyte precursors within the BM that expand in response to exogenous Flt3L.

Lymphocyte development in Flt3L-treated BRGSF HIS mice

We next analyzed development of B and T cells in BRGSF HIS mice receiving or not Flt3L. T cell development as assessed by total thymocyte numbers and CD4/CD8 profiles were unaffected by Flt3L treatment (Figure S3A) and consistent with our previous studies^{18,26,27}. Absolute numbers and phenotype of splenic CD4⁺ and CD8⁺ T cells were not significantly affected by Flt3L treatment, with the majority of cells presenting a naïve CD45RA⁺CD27⁺ phenotype (Figure S3B). BM B cell maturation was similar in BRGSF HIS mice receiving or not Flt3L harboring a predominant CD19⁺ B cell precursors and more mature IgD⁺IgM⁺ B cells (Figure S3C).

Given previous studies demonstrating the impact of DC 'crosstalk' in promoting NK cell maturation and proliferation^{24,37}, we tested whether Flt3L-dependent enhancement of myeloid

cell development in BRGSF HIS mice affected the phenotype and function of conventional NK cells (defined as CD3⁻NKp46⁺CD94⁺ cells). Administration of Flt3L to BRGSF mice led to an increase frequency and absolute number (2- to 2.5-fold) of conventional NK cells in the liver, spleen and lung (Figures 2A, B), as well as in blood, BM and intestine (data not shown). Conventional NK cells in humans include two main subsets: a CD56^{bright}CD16⁻ and a more mature CD56^{dim}CD16⁺ subset (reviewed in ³⁸). As reported in other HIS mouse models^{16,26}, the majority of CD56⁺ NK cells in BRGSF HIS mice were CD16⁻ with about 30% expressing the low affinity Fc receptor (Figure 2C). Flt3L treatment did not change the proportions of CD16⁺ NK cells (Figure 2C), or the expression pattern of the C-type lectin receptors NKG2A and NKG2C (Figure 2D). MHC class I-specific KIR expression was restricted to the CD16⁺ subsets and accounted for 15-30% of the total CD94⁺ population depending on the organ (Figure 2E, left panel). Both activating (KIR2DS4) and inhibitory KIR (KIR2DL1/DL2/DL3) molecules were expressed suggesting a balanced repertoire formation on NK cells. Neither the percentage of KIR-expressing NK cells nor the ratio of activating versus inhibitory receptors was affected by Flt3L treatment (Figure 2E, right panel).

NK cell priming following Flt3L-mediated human DC boost

Because IL-15-transpresentation by DCs has been shown to prime resting NK cells^{39,40}, we compared the cytokine production and degranulation capacity of NK cells in BRGSF HIS mice with or without Flt3L-mediated DC boost. Splenic NK cells were stimulated with IL-12, IL-15 and IL-18 *ex vivo* and IFN-γ production was assessed by intracellular staining (Figure 3A, upper panel). Flt3L treatment increased the frequency of IFN-γ-producing NK cells compared to control mice (Figure 3B). In contrast, NK cell degranulation (CD107a expression) upon cytokine activation was not altered in Flt3L treated mice. In order to assess

DC-NK cell 'crosstalk' *in vivo*, we stimulated DC via TLR3 following i.p. injection of poly(I:C) and assessed NK cell cytokine production *ex vivo* 14 h later. TLR-mediated activation of human DC was confirmed by elevated human IL-12 levels in plasma of mice after poly(I:C) injection in Flt3L boosted BRGSF HIS mice (Figure S4). We found enhanced NK cell IFN- γ production and a marked increase in CD107a expression in response to poly(I:C) activation *in vivo* (Figure 3C). While these responses were also observed in BRGSF HIS mice that had not been Flt3L boosted, the amplitude of the NK cell response was significantly higher in Flt3L treated HIS mice. These results suggest that increased human DC in Flt3L boosted BRGSF HIS mice can be stimulated *in vivo* and can trans-activate effector functions in NK cells.

Administration of Flt3L enhances development of diverse ILC subsets in BRGSF mice

Innate lymphoid cells include several subsets of lineage-CD127⁺ cells that have the capacity to rapidly produce cytokines in the early phases of immune responses (reviewed in ⁴¹). We next assessed the impact of Flt3L treatment in BRGSF mice on the development of ILC (defined as lineage EOMES⁻CD7⁺CD127⁺ cells). ILCs were detected in liver, spleen, lung and gut and accounted for 0.5 to 2% of the total hCD45⁺ cells in these organs (Figure 4A). Administration of Flt3L increased both the ILC frequency (Figure 4A) and absolute numbers (Figure 4B) in all organs tested, with the most pronounced effects in the liver and gut.

Diverse T-BET⁺ IFN- γ -producing ILC subsets have been reported in various locations and with diverse, sometimes overlapping, surface markers (reviewed in ⁴²). We next assessed ILC1 heterogeneity in BRGSF mice, focusing on the liver, where several CD127⁺ ILC1 populations distinct from NK cells have been reported⁴³⁻⁴⁵. BRGSF mice harbor two T-BET⁺ lineage⁻CD7⁺ populations that can be distinguished on the basis of IL7R α (CD127)

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expression (Figure 5A). T-BET⁺CD127⁻ cells represent conventional NK cells and co-express EOMES, CD161 and high levels of CD94 (Figure 5B). In contrast, a second T-BET⁺CD127⁺ subset could be detected that also expressed CD161 and EOMES but showed an essentially negative expression of CD94 (Figure 5B). These T-BET⁺CD127⁺ cells did not express the NK marker CD16 and had lower expression of NKp46 than conventional NK cells (Figure 5C). This ILC1 subset accounted for less than 0.1% of hCD45⁺ liver cells, similar to a recently described human intrahepatic NK-like ILC⁴⁶. Flt3L treatment increased the frequency and absolute numbers of these hepatic ILC1 (Figure 5D).

We next addressed the IFN- γ production capacity of Lin⁻CD7⁺T-BET⁺ cells upon *ex vivo* stimulation. Both T-BET⁺CD127⁺ ILC1 and conventional T-BET⁺CD127⁻ NK cells showed strong IFN- γ production after pharmacological activation (Figure 5E). We further showed that CD161⁺ ILC1 could be activated following TLR triggering *in vivo* (using a TLR-4, -5, -7 and -8 ligand cocktail of flagellin, LPS and R484). Flt3L-treated BRGSF HIS mice were TLR-stimulated treated or not and 16 h later hepatic lymphocytes were stained for intracellular IFN- γ . We found that TLR stimulation was able to stimulate an IFN- γ response in CD161⁺ liver ILCs above levels observed in non-TLR-stimulated mice (Figure 5F) suggesting that DC activation via TLR stimulation is able to trans-activate hepatic ILC1 *in vivo*.

Increased lung ILC2 homeostasis in Flt3L treated BRGSF HIS mice

We next studied the impact of Flt3L treatment on other ILC subsets. ILC2 are present at mucosal sites and play a key role in barrier protection in the respiratory tract (reviewed in ⁴⁷). We detected human ILC2 in the lung of BRGSF HIS mice as a population of lineage⁻ CD7⁺CD127⁺T-BET⁻EOMES⁻GATA-3⁺ cells (Figure 6A). The frequency and absolute numbers of ILC2 within the lung of BRGSF HIS mice was increased by Flt3L treatment

(Figure 6B). On average, GATA-3⁺ ILC2 represented about 0.35% of the human CD45 cells within the lung.

We found that ILC2 in the lungs of BRGSF HIS mice had phenotypic similarities to ILC2 from human tissues^{9,48–50}, with notable expression of the prostaglandin D2 receptor CRTh2, c-kit receptor (CD117) and the chemokine receptor CCR6 and low or negative expression of CD56 (Figure 6C). Lung ILC2 in BRGSF HIS mice showed detectable IL-13 production under steady-state conditions that could be increased after *ex vivo* stimulation with PMA plus ionomycin (Figure 6D). In order to assess the impact of survival factors and inflammatory cytokines on human ILC2 function *in vivo*, we administrated *in vivo* a cocktail of human cytokines (IL-2, -7, -25 and -33) by hydrodynamic injection and assessed IL-13 production capacity six days later. Following *in vivo* cytokine expansion and stimulation, we found that a substantial proportion of ILC2 expressed IL-13 (Figure 6D). These results suggest that human ILC2 in BRGSF HIS mice are cytokine responsive *in vivo* and that ILC2 function can be primed under appropriate environmental conditions.

Flt3L stimulates intestinal ILC homeostasis and function in BRGSF HIS mice

ILC3 are enriched in intestinal lamina propria and play a fundamental role in gut homeostasis and immune defense. Human CD45⁺ cell engraftment in the gut was quite variable in individual BRGSF HIS mice (Figure 7A) ranging from non-reconstitution to $6x10^{6}$ hCD45⁺ cells in intestinal lamina propria. In reconstituted HIS mice, gut lamina propria cells included T cells (4-20%), CD94⁺ NK cells (3-15%) and a clearly detectable subset of lineage CD7⁺CD127⁺ ILC (1-5%). These intestinal ILCs expressed CD117 but were negative for NKp46 (Figure 7D) and therefore resembled NCR⁻ ILC3 previously described in diverse human tissues^{43,51}. Flt3L administration resulted in an increase of the frequency of gut ILC3 (reaching in some cases 10% of the total hCD45⁺ cell population) that was accompanied by an increase in total ILC number (Figures 7B, C). Intestinal ILC3 in BRGSF HIS mice expressed the transcription factor RORγt and following *in vitro* simulation, were able to produce IL-22 and IL-17A (Figure 7E). When human DC were stimulated *in vivo* with a cocktail of TLR ligands (see above), we found that intestinal ILC3 showed a modest increase of IL-22 production with no impact of the Flt3L treatment (Figure 7F). However, IL-17A was drastically boosted after stimulation and that increase was even more prominent in the Flt3L treated mice suggesting a positive effect of the ligand on the functional fitness of ILC3 *in vivo*.

Discussion

Innate lymphoid cells play fundamental roles in the early immune response against diverse pathogens and in later stages of infection and inflammation through promotion of tissue homeostasis and repair. As such, ILC activation and regulation may impact upon disease outcome in some clinical conditions. The development of suitable small animal models that recapitulate ILC development and function *in vivo* could provide a preclinical testing platform to assess immunomodulatory approaches that target ILCs. Here we show that Flt3L-boosted BRGSF recipients provides a simple HIS mouse model for robust generation of diverse human innate and adaptive lymphocyte subsets. In particular, we find that mature, functional human ILC subsets are present in lymphoid and non-lymphoid tissues of BRGSF HIS mice thereby opening a path to understand human ILC immunobiology *in vivo* in the context of infection, inflammation and cancer.

Two non-exclusive mechanisms may underlie the ability of Flt3L-boosted BRGSF HIS mice to robustly develop human ILCs. As Flt3L regulates early DC precursors and DC homeostasis^{22–25,37} (Figure 1, S2), an improved myeloid compartment may provide a richer human cytokine environment⁵² thus indirectly supporting ILC survival and proliferation. This mechanism underlies DC-transpresentation of IL-15 to mouse and human NK cells^{15,24,39}. A second mechanism may involve direct effects of Flt3L on CD135⁺ lymphoid committed precursors that are upstream of human ILC or NK precursors⁵³⁻⁵⁵. These two pathways may act synergistically to contribute to the observed human ILC boost in BRGSF HIS mice.

NK cells engage in bidirectional interactions with other innate effectors that modulate their differentiation, homeostasis and immune responses against pathogens and cancer⁵⁶. This innate 'crosstalk' can further impact adaptive responses as evidenced by the reduced benefit of DC vaccination after NK cell depletion in a melanoma mouse model⁵⁷. Both soluble (IFN- γ) as well as contact-dependent (OX40-OX40L) factors contribute to NK cell –

DC immune orchestration. These interactions may underlie the increased cytokine production and degranulation capacity we observe in BRGSF mice. As both myeloid and NK cells are expanded in BRGSF HIS mice, our model provides an opportunity to study the importance of this crosstalk in human innate immunity.

The robust ILC development we observed in BRGSF HIS mice suggests that ILC homeostasis may be regulated by similar Flt3L-dependent mechanisms as found for human NK cells. We observed evidence for phenotypic diversity within the IFN- γ -producing ILCs in the liver of BRGSF mice, with T-BET⁺ cells heterogeneously expressing CD127 and CD94 that finds parallels with recent reports in humans^{19,46}. While the environmental factors that regulate human ILC1 diversity are not clearly identified, our BRGSF HIS mouse model provides a means to dissect ILC1 heterogeneity in tissue environments under steady-state and inflammatory conditions.

We identified functional GATA-3⁺ human ILC2 in the lungs of BRGSF HIS mice. Previous studies have not yet described human ILC2 in humanized mice, and as such, the BRGSF model is therefore unique in this regard. ILC2 play critical roles in immune protection against various pathogens (helminths, viruses) and are associated with atopic diseases and reactive airways (reviewed in ⁵⁸). Moreover, ILC2 have been recently shown to have functional plasticity in the context of inflammation^{49,59,60}. BRGSF HIS mice may provide a valuable new model to study the biology of human ILC2 *in vivo* in the context of inflammation.

We observed that BRGSF HIS mice support robust human immune reconstitution in the mouse intestine, including RORyt⁺ ILC3. ILC3 play an important role in maintaining barrier function (reviewed in ⁶¹). Human intestinal ILC3 protect against colitis through production of IL-22 and regulate T cells by MHC call II mediated presentation of microbial antigens⁶², a process that may be modeled in humanized mice⁶³. Our BRGSF HIS mouse model may

provide a means to dissect the biology of human intestinal immune tolerance. A recent report showed that HIV-1 infection depletes human ILC3 thus providing a plausible mechanism for loss of intestinal homeostasis in the context of this disease²⁰. Enhanced human ILC3 development in BRGSF HIS mice offer a means to study these cells in various viral inflammation conditions.

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Authorship

Contribution: S.L.L., G.M.R., O.F., S.D., N.S., M.D., H.S.-M. and Y.L. performed experiments and provided essential expertise. S.L.L. and J.P.D. designed research and wrote the manuscript.

Conflict-of-interest disclosure: J.P.D. is a stakeholder in AXENIS (founder, member of the executive board). The remaining authors declare no conflict of interest.

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Figure Legends

Figure 1: Distribution of human myeloid subsets in BRGSF mice and effect of Flt3L on their development. Representative flow cytometry immuno-phenotypic analysis of hCD45⁺HLA-DR⁺CD19-CD3-CD56- cells from bone marrow (A) and spleen (C) of a Flt3L treated mouse and a PBS treated littermate engrafted with the same CD34⁺ HSC donor. Comparison of frequencies within the human CD45 positive cells and total number of the four myeloid subsets (CD14⁺ monocytes, CD123⁺ pDC, CD141⁺ cDC and CD1c⁺ cDC) with or without Flt3L treatment in bone marrow (B) and spleen (D). Each dot represents one mouse. Composite data from 3 independent experiments are shown. Numbers in plots represent frequencies within gates.

Figure 2: Distribution of human NK cells in reconstituted BRGSF mice with or without Flt3L treatment. A) Representative flow cytometry immuno-phenotypic analysis of alive hCD45⁺CD3-CD94⁺NKp46⁺ NK cells from liver, spleen and lung of a Flt3L treated mouse and a PBS treated littermate engrafted with the same CD34⁺ HSC donor. B) Comparison of CD94⁺ cell frequencies within the human CD45 positive cells (upper graph) and total number of NK cells (lower graph) with or without Flt3L treatment in liver, spleen and lung. C) Representative flow cytometry plot of CD56 and CD16 expression in liver NKp46⁺CD94⁺ cells as gated in top panels (left) and comparative quantification (right). D) Expression of NKG2A and NKG2C in liver NKp46⁺CD94⁺ cells (left) and comparative quantification (right). E) Distribution of activating (CD158i) and inhibitory (CD158a/b) KIR expression in liver, spleen and lung NK cells of a representative BRGSF mouse treated with Flt3L (left plots) and comparative quantification of the total KIR expressing CD94⁺ NK cells with or without Flt3L treatment (right graph). Each dot represents one mouse. Composite data from at least three independent experiments are shown. Numbers in plots represent frequencies within gates.

Figure 3: Flt3L treatment enhances human NK-cell function in spleen cells from reconstituted BRGSF mice. A) Human NK cells were MACS enriched from spleens of BRGSF Flt3L treated or not and were stimulated *ex vivo* with the monokines IL-12, IL-15 and IL-18. Representative flow cytometry immuno-phenotypic analysis of degranulation (CD107a) and cytokine production (IFN γ) in NKp46⁺ NK cells is shown. B) Quantification of IFN γ -producing and CD107a-expressing NK cells from Flt3L treated or control BRGSF mice. C) *In vivo* functionality of NK cells in BRGSF mice was evaluated by quantifying IFN γ production and degranulation after *in vivo* poly (I:C) stimulation. Panel B and C represent composite data from of 4 mice per condition in 2 experiments. Each dot represents one mouse. Numbers in plots represent frequencies within gates.

Figure 4: Distribution of human ILCs in BRGSF mice and effect of Flt3L treatment. A) Representative flow cytometry analysis of Lin⁻EOMES⁻CD7⁺CD127⁺ innate lymphoid cells in spleen, liver and lung and Lin⁻CD94⁻CD7⁺CD127⁺ cells in gut of a Flt3L treated BRGSF mouse. B) Comparative quantification of ILCs in spleen, lung, liver and gut of BRGSF mice treated or not with Flt3L as a percentage of hCD45⁺ cells (upper panel) and total number of cells (lower panel). Composite data from of 14 mice per condition in 4 experiments. Each dot represents one mouse. Numbers in plots represent frequencies within gates.

Figure 5: Flt3L treatment expands a population of ILC type 1 cells different from NK cells. A) Representative flow cytometry analysis of T-BET and CD127 expression on Lin⁻ CD7⁺ cells in liver of a PBS and a littermate Flt3L treated BRGSF mouse. B) Expression of CD117, EOMES, CD161 and CD94 (histograms) on liver T-BET⁺CD127⁻ (red), T-

BET⁺CD127⁺ (blue) and T-BET⁻CD127⁺ cells (grey) assessed by flow cytometry. C) Expression of CD127, CD16 and NKp46 on liver Lin⁻T-BET⁺ cells, assessed by flow cytometry. D) Frequency among hCD45⁺ cells (left panel) and total number (right panel) of Lin⁻CD7⁺CD127⁺T-BET⁺ liver cells. E) Representative functional analysis by IFN- γ intracellular flow cytometry after P/I + cytokine 4h stimulation of T-BET⁺CD127⁻ (red) and T-BET⁺CD127⁺ (blue) cells. Gated were determined using unstimulated controls. F) Flow cytometry representation of IFN- γ production by Lin⁻T-BET⁺CD161⁺ ILC1 cells in response to TLR-mediated *in vivo* stimulation. Composite data from of 3-4 mice per condition is shown in left panel. Numbers in plots represent frequencies within gates.

Figure 6: Flt3L treatment also augments ILC type 2 cells in reconstituted BRGSF mice.

A) Representative flow cytometry analysis of GATA-3⁺ ILCs in lung of reconstituted BRGSF treated or not with Flt3L. B) Frequency among hCD45⁺ cells and total number of GATA-3⁺ ILCs in lung of reconstituted BRGSF treated or not with Flt3L. Composite data from 8 mice of 2 experiments is shown C) Representative functional analysis by IL-13 intracellular flow cytometry from freshly isolated lung ILCs, after P/I *ex vivo* stimulation and after *in vivo* hydrodynamic cytokine injection and *ex vivo* stimulation. D) Expression of CRTh2, CCR6, CD56 and CD117 (histograms) on lung GATA-3 ILCs (red) and EOMES⁺T-BET⁺ NK cells (grey) assessed by flow cytometry. Each dot represents one mouse. Numbers in plots represent frequencies within gates.

Figure 7: ILC3 can be found in the gut of BRGSF reconstituted mice and their frequency increase after Flt3L treatment. A) Total human CD45 cell number in gut of BRGSF mice treated or not with Flt3L. Composite data from of 12-16 mice per group is shown. B) Representative flow cytometry analysis of ILCs in gut of reconstituted BRGSF

treated or not with Flt3L. C) Frequency among hCD45⁺ cells and total number of ILCs in total gut of reconstituted BRGSF treated or not with Flt3L. Composite data from 6/8 mice of 2 experiments is shown. Each dot represents one mouse. D) ILC (Lin⁻CD94⁻CD7⁺CD127⁺) (in red) expression of CD117 and NKp46 as compared to NK cells (grey). E) Intracellular expression of RORγt in gut ILCs of a Flt3L treated mouse (top panel) and representative functional analysis by IL-22 and IL-17 intracellular flow cytometry from *ex vivo* stimulated ILCs. F) Quantification of IL-22 and IL-17 production analyzed by flow cytometry from Lin⁻ CD94⁺CD7⁺CD127⁺ ILC3 cells in response to TLR-mediated *in vivo* stimulation. Composite data from of 3-4 mice per condition is shown. Numbers in plots represent frequencies within gates.

Supplemental Figure Legends

Supplemental Figure 1: Evaluation of circulating human hematopoietic cells in blood of BRGSF mice 10 weeks post-engraftment. A) Frequency of human leukocytes (hCD45⁺) in blood 10 weeks post-engraftment of BRGSF mice. B) Representative flow cytometry analysis showing reconstitution of the main blood cell populations within the hCD45 compartment. CD3⁺ T cells, CD19⁺ B cells, CD3⁻NKp46⁺ NK cells and CD3⁻CD19⁻ CD11c⁺HLA-DR⁺ DCs develop in BRGSF mice prior in absence of any treatment.

Supplemental Figure 2: Flt3L treatment augments myeloid precursor populations in the BM of BRGSF mice. A) Frequency of total human CD34⁺ hematopoietic stem cells in bone marrow of BRGSF mice and effect of Flt3L treatment. B) Representative flow cytometry gating strategy for the analysis of Lin⁻CD34⁻CD117⁺CD135⁺CD116⁺CD45RA⁺DC/monocyte precursors in BM of BRGSF mice. C) Frequency (left) and total number (right) of human

myeloid hematopoietic precursor in bone marrow of BRGSF mice and effect of Flt3L treatment.

Supplemental Figure 3: The distribution and phenotype of adaptive lymphocytes is not affected by Flt3L treatment in BRGSF mice 10 weeks after HSC reconstitution. A) Relative proportions of major thymocyte maturation stages: double negative (CD4⁺CD8⁺), double positive (CD4⁺CD8⁺) and single positive CD4⁺ and CD8⁺ cells in BRGSF thymus and the effect of Flt3L treatment. B) Distribution of CD4 and CD8 T subsets within the CD3⁺ cells (left) and differentiation status of CD8⁺ (middle) and CD4⁺ T cells (right) as for central memory (CD45RA⁻CD27⁺), naïve (CD45RA⁺CD27⁺), effector memory RA (CD45RA⁺CD27⁻) and effector memory (CD45RA⁻CD27⁻). C) Frequency among hCD45⁺ cells of CD19⁺, CD19⁺CD20⁺ and IgD⁺IgM⁺ B cells in bone marrow of BRGSF with or without Flt3L treatment. Composite data from of 4 mice per condition is shown.

Supplemental Figure 4: Poly I:C treatment leads to increased IL-12 levels in plasma of HIS mice. Plasma levels of IL-12 were determined by Luminex technology in plasma of BRGSF mice prior to the Flt3L treatment, after the six treatment doses (before the poly (I:C) stimulation) and at the moment of the analysis. Composite data of four mice per group is shown.

Supplemental Table 1: Antibodies used for flow cytometry analysis







o PBS

Fit3L

÷

CD107a

PBS
Flt3L

polyl:C















Figure S1











Figure S4



Supplemental Table 1

ANTIBODY	CLONE	COMPANY	CATALOG#
Anti-human CD141 BV711	1A4 (RUO	BD Bioscience	563155
Anti-human CD1c PB	L161	Biolegend	331508
Anti-human CD33 APC	AC104.3E3	Miltenyi Biotec	130-091-731
Anti-human CD123 PerCP-Cy5.5	6H6	eBioscience	45-1239-73
Anti-human CD11c PE-Cy7	3.9	eBioscience	25-0116-42
Anti-human CD14 PE-CF594	ΜΦΡ9	BD Bioscience	562335
Anti-human HLA-DR APC-Cy7	L243	Biolegend	307618
Anti-human CD3 FITC	UCHT1	eBioscience	11-0038
Anti-human CD45 BV506	HI30	Biolegend	304042
Anti-human CD94 APC-Vio770	REA113	Miltenyi Biotec	130-091-641
Anti-human NKp46 BV421	9-E2	BD Bioscience	564065
Anti-human CD56 AF700	B159	BD Bioscience	557919
Anti-human CD16 APC	B73.1	BD Bioscience	561304
Anti-human CD159a FITC	REA110	Miltenyi Biotec	130-098-818
Anti-human CD159c PE	134591	R&DSystems	FAB138P-100
Anti-human CD158a APC	REA284	Miltenyi Biotec	130-103-966
Anti-human CD158b APC	DX27	Miltenyi Biotec	130-092-617
Anti-human CD158i PE-Cy7	JJC11.6	Miltenyi Biotec	130-099-963
Anti-human CD107a FITC	eBioH4A3	eBioscience	12-1079-71
Anti-human IFNy FITC	45-15	BD Bioscience	563580
Anti-human IL-13 BV421	JES10-5A2	eBioscience	25-7229
Anti-human IL-22 PE-Cyanine7	22URTI	Miltenyi Biotec	130-094-519
Anti-human IL-17A APC	CZ8-23G1	BD Bioscience	564358
Anti-human CD5 FITC	UCHT2	eBioscience	11-0059
Anti-human CD7 PE-CF594	M-T701	BD Bioscience	562541
Anti-human CD127 PE-Cyanine 7	RDR5	eBioscience	25-1278
Anti-human EOMES PE	WD1928	eBioscience	12-4877-42
Anti-human T-BET BV421	4B10	Biolegend	644816
Anti-human/mouse GATA-3 PerCP-eFluor 710	TWAJ	eBioscience	46-9966
Anti-human/mouse RORgamma (t) APC	AFKJS-9	eBioscience	17-6988
Anti-Human CD161 PerCP-Cyanine5.5	HP-3G10	eBioscience	45-1619-42
Anti-human CD117 BV605	104D2	Biolegend	313218
Anti-human CRTh2-PE	BM16	Miltenyi Biotec	130-098-238
Anti-human CD196 PerCP-Cy5-5	11A9	Biolegend	354514
Anti-human NKp44 BB15/AF647	p44-8	BD Bioscience	565099/558564
Anti-human CD34 APC	581	BD Bioscience	555824
Anti-human CD135 PE	4G8	BD Bioscience	558996
Anti-human CD116 BV711	hGMCSFR-M1	BD Bioscience	743380
Anti-human CD45RA VioBlue	T6D11	Miltenyi Biotec	130-095-464
Anti-human IgD PerCP-Cy5-5	IA6-2	BD Bioscience	561315
Anti-human IgM FITC	MHM-88	Biolegend	314506
Anti-human CD20 PB	2H7	Biolegend	302328
Anti-human CD8 PE-CF594	RPA-T8	BD Bioscience	562282
Anti-human CD4 FITC	OKT4	eBioscience	11-0048
Anti-mouse CD45.1 FITC	A20	Miltenyi Biotec	130-102-457
Anti-mouse CD45.2 FITC	104.2	Miltenyi Biotec	130-102-458

PAPER II: Systemic human ILC precursors provide a substrate for tissue ILC differentiation

Human circulating ILC can be identified as a low frequency population within lineage⁻CD7⁺CD56⁻CD127⁺ peripheral blood cells of healthy individuals as well as patients suffering from diverse clinical syndromes. Further fractionation into ILC1, ILC2 and ILC3 has been achieved using the phenotypic markers mentioned previously in this manuscript by several groups. Circulating ILCs also include a predominant CD117⁺ subset that lacks CRTh2 expression and has been previously considered circulating ILC3 since tissue-resident ILC3 strongly express CD117. However, we found that PB CD117⁺ ILC differ dramatically from gut CD117⁺ ILC in that they lack expression of NKp44 and other surface markers associated to ILC3s and the transcription factor RORγt and do not produce IL-17A or IL-22 after stimulation.

Here, we used <u>BALB/c</u> <u>Rag2</u>^{-/-}*Il2rg*^{-/-}<u>Sirpa</u>^{NOD} (BRGS) to analyze the lineage potential of this CD117⁺ subset. While BRGS mice engrafted with HSCs showed multilineage reconstitution, those receiving PB CD117⁺ ILCP developed Lin⁻CD7⁺ cells but no myeloid cells, B cells or T cells. Human CD45⁺ progeny from transferred CD117⁺ ILCP were detected in multiple organs, including the spleen, lung, gut and liver. At each of these tissue sites, EOMES⁺ NK cells as well as diverse CD127⁺ ILC subsets could be identified that produced IFN- γ ILC subsets could be identifiex vivo upon stimulation.

This ILC precursor population was also found in BRGS mice engrafted with CD34⁺ HSCs. Paralelling human PB ILCP, these cells failed to elicit cytokine production after *ex vivo* stimulation. However *in vitro* culture in the presence of IL-2, IL-7, IL-1 β led to the expansion of cells able to produce IFN- γ , IL-13, IL-17A and IL-22 thereby confirming the presence of human ILCP in HIS mice. These results demonstrate that CD34⁺ HSC can give rise to CD117⁺ ILCP *in vivo* validating the BRGS HIS mouse model as a translational platform to study human hematopoiesis.

Systemic human ILC precursors provide a substrate for tissue ILC differentiation

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Summary

Innate lymphoid cells (ILC) represent innate versions of T helper and cytotoxic T cells that differentiate from committed ILC precursors (ILCP). How ILCP give rise to mature tissue-resident ILC remains unclear. Here we identify circulating and tissue-resident ILCP in humans that fail to express the transcription factors and cytokine outputs of mature ILCs but have these signature loci in an epigenetically poised configuration. Human ILCP robustly generate all ILC subsets *in vitro* and *in vivo*. While human ILCP express RAR related orphan receptor C (RORC), circulating ILCP can be found in RORC-deficient patients that retain potential for EOMES⁺ NK cells, IFN- γ^+ ILC1, IL-13⁺ ILC2 and for IL-22⁺ but not for IL-17A⁺ ILC3. These results support a model of tissue ILC differentiation ('ILC-poiesis') whereby diverse ILC subsets are generated *in situ* from systemically distributed ILCP in response to local environmental signals.

Introduction

Innate lymphoid cells (ILC) are a novel family of lymphoid effector cells that serve essential roles in the early immune response, consisting of cytotoxic cells (NK cells) and 'helper-like' ILC. The latter are characterized by expression of interleukin-7 receptor (IL-7R α /CD127) and categorized into three distinct groups based on their transcription factors (TF) and signature cytokine production. Group 1 ILC (ILC1) express TBX21 (encoding T-BET) and produce T helper (T_H)1-associated cytokines IFN- γ and TNF- α . Group 2 ILC (ILC2) express GATA-3 and RORA and secrete T_H2-associated cytokines, IL-5 and IL-13. Group 3 ILC (ILC3) utilize RORC (encoding ROR γ t) to drive production of the T_H17-associated cytokines, IL-17 and/or IL-22 (Serafini et al., 2015; Spits et al., 2013). These different ILC subsets are found in diverse lymphoid and non-lymphoid tissues, and enriched at mucosal sites where they play essential roles in barrier function and innate immune defense (Artis and Spits, 2015; Eberl et al., 2015).

Diverse human ILC subsets were first identified in secondary lymphoid tissues and subsequently reported at several non-lymphoid tissue sites (intestine, lung, liver, skin) (reviewed in (Juelke and Romagnani, 2016)). Two distinct populations of IFN- γ -producing ILC1 have been described. A T-BET⁺ cell expressing high levels of CD127 and CD161 but lacking other specific surface markers has been identified in tonsil and inflamed intestine (Bernink et al., 2013). In contrast, an intraepithelial ILC1 expressing NKp44 and CD103 but not CD127 resides at mucosal sites (Fuchs et al., 2013). Both these ILC1s produce IFN- γ in response to IL-12 but unlike NK cells they show minimal EOMES expression. Human GATA-3⁺ ILC2 express the chemoattractant receptor CRTh2 as well as IL-25R and IL-33R (Mjösberg et al., 2011), are widely distributed (lung, skin, gut, nasal polyp, adipose tissues) and produce type 2 cytokines IL-5 and IL-13 under a variety of physio- and pathological situations (reviewed in (Kim and Artis, 2015)). Group 3 ILC include ROR γ t⁺ fetal lymphoid tissue-inducer (LTi) cells identified in fetal mesenteric lymph nodes and spleen (Cupedo et al., 2009) as well as adult lineage⁻CD127⁺CD117⁺ cells found in adult tonsils, intestine, spleen, skin, lung, endometrium and decidua (reviewed in (Klose and Artis, 2016)). A subset of ILC3 express natural cytotoxicity receptors (NCR, including NKp30, NKp44 and NKp46) and are enriched in IL-22-producing cells (Cella et al., 2009; Satoh-Takayama et al., 2008).

Murine mature ILC differentiate from hematopoietic stem cells (HSC) via a common lymphoid progenitor (CLP) to give rise to diverse ID2⁺TCF-1⁺PLZF⁺ ILC precursors (ILCP) in fetal liver (FL) and adult bone marrow (BM) (Constantinides et al., 2014; Yang et al., 2015). Several TF and signaling pathways regulate this process in mice (Serafini et al., 2015); in contrast, human ILC development is less well characterized (Juelke and Romagnani, 2016)). NK precursors (NKP) have been identified in FL, BM, cord blood (CB) and adult tonsil (Renoux et al., 2015), whereas committed ILC3 precursors (ILC3P) are found in tonsil and intestinal lamina propria but not peripheral blood (PB), thymus or BM (Montaldo et al., 2014). A recent study identified tonsillar human ROR γ t⁺ ILCP that can develop into mature cytotoxic and helper ILC (Scoville et al., 2016). Interestingly, these human NKP, ILC3P and ILCP were CD34⁺ and enriched in secondary lymphoid tissues but were rare or absent from the circulation. It was unclear how these CD34⁺ hematopoietic precursors were developmentally related to mature tissue-resident ILC subsets.

In this report, we have extensively characterized the phenotypic, molecular and functional attributes of peripheral blood and tissue-resident CD117⁺ ILCs. This included an *in vitro* study of over 1300 ILC clonal cultures as well as an *in vivo* analysis using humanized mice. While ILCs with the CD117⁺ phenotype were previously proposed to represent human ILC3 (Hazenberg and Spits, 2014), we find that peripheral blood CD117⁺ cells are remarkably enriched in multi-potent and uni-potent ILC precursors (ILCP) that can give rise *in vitro* and *in vivo* to all known ILC subsets, including EOMES⁺ NK cells. CD117⁺ ILCP are

found not only in the circulation, but also in tissues where they retain ILC multipotency. Our identification of systemically distributed ILCP suggests a model whereby circulating and tissue-resident ILCP provide a cellular substrate for ILC differentiation *in situ* in response to local environmental signals.

Results

Characterization of human peripheral blood CD117⁺ ILC

Circulating ILC can be identified as a low frequency population (< 0.2% of total CD45⁺ cells) within lineage⁻⁻CD7⁺CD56⁻⁻CD127⁺ peripheral blood (PB) cells of healthy individuals as well as patients suffering from diverse clinical syndromes ((Hazenberg and Spits, 2014; Munneke et al., 2014); Figure 1A and 1B). Further fractionation of PB ILCs into ILC1, ILC2 and ILC3 has been achieved using phenotypic markers that distinguish ILC subsets in fetal tissues and tonsils, including CD161, CRTh2, CD117 and NKp44 (Spits et al., 2013). Circulating ILCs include predominant CRTh 2^+ ILC2 and a CD117⁺ subset that lacks CRTh2 expression (Figure 1A and 1B). While CD117⁺ cells are widely considered as circulating ILC3 (Hazenberg and Spits, 2014; Munneke et al., 2014), we found that PB CD117⁺ ILC differ dramatically from gut CD117⁺ ILC in that they lack expression of NKp44 and RORyt that identifies ILC3 (Figure 1C and S1A). Accordingly, PB CD117⁺ ILC do not produce IL-17A or IL-22 after pharmacological stimulation, whereas gut CD117⁺ cells abundantly produce these ILC3-associated cytokines (Figure 1D). Interestingly, circulating CD117⁺ ILC express high levels of IL-1R1, CD45RA and are CD69⁻⁻, whereas gut-resident ILC3 are CD69⁺ but IL-1R1⁻ and CD45RA⁻ (Figure 1C and S1B). These observations suggest that PB CD117⁺ ILC differ from *bona fide* ILC3.

PB CD117⁺ ILC also do not express TF signatures that characterize other known ILC subsets (Figure 1C). Accordingly, PB CD117⁺ ILC failed to express markers associated with NK cells, ILC1 and ILC2, such as CD94, CD244, CRTh2 (Figure S1B) and did not produce IFN- γ or IL-13 after stimulation with pharmacological activators (Figure 1D). Taken together, these results show that PB CD117⁺ ILC may represent a novel ILC subset.

Transcription and chromatin landscapes of CD117⁺ ILC reveal an ILC precursor profile

In order to further understand the identity of PB CD117⁺ ILC, we profiled the transcriptomic and epigenetic landscapes of highly purified circulating CD117⁺ ILC compared to CD34⁺ HSC (Figure 2A). We performed chromatin immunoprecipitation followed by high-throughput sequencing using transposase-mediated tagmentation (ChIPm-Seq; (Schmidl et al., 2015). To expose common and unique epigenetic features of CD34⁺ HSC and CD117⁺ ILC, we mapped histone H3 lysine 4 di-methyl modifications (H3K4Me2), which marks both active and poised gene regulatory elements (GRE) (Koche et al., 2011). We identified around 18,000 and 35,000 GRE in CD117⁺ ILC and CD34⁺ HSC respectively (Figure 2B), the majority of which were located in introns and intergenic regions (Figure S2A). A significant number of H3K4Me2⁺ GRE were shared between the two cell types: 89% of GRE identified in CD117⁺ ILC showed similar enrichment in HSC and were associated with 13159 genes of which many encoded housekeeping functions. Nevertheless, 11% of H3K4Me2⁺ GRE detected in CD117⁺ ILC were absent in CD34⁺ HSC, potentially regulating 2283 genes. Pathway analysis of these genes revealed a strong enrichment for immune system and lymphocyte related processes. For example, cytokine/chemokine signaling genes critical for lymphoid development and function such as IL1R1, IL7R, IL2RA/B were linked to a CD117⁺ ILC-specific GRE. Conversely, GRE only active in CD34⁺ HSC (54% of all GRE in CD34⁺ HSC) were located near genes involved in more general pathways important for hematopoiesis, hemostasis, and platelet activation (Figure 2C).

To compare the transcriptome of CD117⁺ ILC and CD34⁺ HSC, we performed RNA sequencing (RNA-Seq). Clear differences in gene expression profiles emerged, with a large cluster of 1540 genes expressed at substantially higher levels in CD117⁺ ILC (Figure 2D). Among these were many genes strongly linked to the lymphoid lineage, including *IKZF1*, *CD2*, *CD7 and IL7R*. In contrast, CD34⁺ HSC cells highly expressed genes involved in the broader development of diverse hematopoietic lineages, including *ID1*, *GATA1*, *GATA2* and

MYB (Figure 2D and S2B) as well as cytokine receptors for myeloid lineages (*CSF3R*, *CSF2RB*, *FLT3*). Compared to HSC, CD117⁺ ILC express high levels of TF that have been shown to be essential for murine ILC development, including *ID2*, *GATA3*, *TOX* and *TCF7*. We did not detect transcripts characteristic of T and B cells development, such as *RAG1*, *RAG2*, *EBF1*, *CD3E*, *BCL11A* or *LMO2* in CD117⁺ ILC although some of these genes are expressed by CD34⁺ hematopoietic progenitors (Figure S2B).

As both transcriptomic and epigenetic analyses of CD117⁺ ILC identified strong lymphoid signatures, we intersected these datasets in order to gain insights into the developmental status and potential of CD117⁺ ILC. A substantial proportion (26%) of the genes most highly expressed in CD117⁺ ILC were located in the direct vicinity of a CD117⁺ ILC-specific GRE (Figure 2E). These included many transcription factors previously implicated in mouse ILC development, including *ID2, GATA3, ETS1, TOX, TCF7, RORA* and *NOTCH1* (Figure 2E and S2A) – consistent with the commitment of CD117⁺ ILC to the innate lymphoid fate. In contrast, we did not detect notable expression levels for mature ILC TF (*EOMES, TBX21, RORC*), cytokine receptors (*CCR6, IL1RL1, IL23R*) or signature cytokines (*IFNG, IL13, IL5, IL22, IL17A*). However, many of these mature ILC identity genes were already marked with H3K4Me2, demonstrating that they may reside in a poised state (Figure 2F and S2B). Together, these analyses suggest that CD117⁺ ILC represent lymphoid-biased progenitors reminiscent of multi-potent ILCP carrying a TF profile with key mature ILC signature genes in an epigenetically poised state.

Peripheral blood CD117⁺ ILC include multi-potent ILC precursors (ILCP)

In order to assess the hematopoietic potential of circulating CD117⁺ ILC, we cultured these cells in the presence of various cytokines. As CD117⁺ ILC express CD25, CD127 and IL-1R1 (Figures 1A, 1C and S1B), we added IL-2, IL-7 and/or IL-1 β to these cultures. While

bulk cultures minimally expanded in the presence of IL-2 and IL-7, robust proliferation was observed when cells were cultured in IL-1 β . The additional presence of cytokines that can drive ILC1/NK (IL-12, -18), ILC2 (IL-25, -33) or ILC3 (IL-23) development did not further increase cell yield over that obtained with IL-1 β (Figure 3B; data not shown). Cultured cells did not harbor B (CD19⁺) or T (CD3⁺CD5⁺) cells but comprised a pure population of CD7⁺ cells that were CD161⁺ and expressed variable levels of CD117 and CD25 (Figure 3A). Remarkably, expanded cells included some EOMES⁺CD94⁺ NK cells as well as the three canonical ILC groups: IFN- γ^+ ILC1, IL-13⁺ ILC2 and NKp44⁺IL-17A⁺IL-22⁺ ILC3 (Figure 3A). The addition of IL-12 clearly promoted the development of EOMES⁺CD94⁺ IFN- γ -producing NK cells and IL-23 was critical for IL-17A-producing-ILC3 (Figure 3A and S3A). These results not only define a cytokine millieu that supports multi-lineage ILC and NK cell generation (IL-2, -7, -1 β , -23) but also suggest that the PB CD117⁺ ILCs harbor multi-potent ILC precursors (ILCP).

We further characterized circulating CD117⁺ ILCP using a modified stromal cellbased culture system that is permissive for B cell, T cell and myeloid cell development (Figure 3C, (Mohtashami et al., 2010)). Moreover, this system can extensively expand human NK cells and ILC subsets at the clonal level with minimal plasticity (Lim et al., 2016). We analyzed progeny of single PB CD117⁺ ILC cultured on OP9 and OP9-DL4 to identify EOMES⁺ NK cells and ILC subsets producing IFN-γ, IL-13, IL-17A and/or IL-22 (Figure 3D). Our analysis of over 340 clonal cultures allows several points to be made. First, PB CD117⁺ ILC represent a heterogeneous population of uni-potent and multi-potent ILCP. Roughly half of the cultures derived from single CD117⁺ ILC generated a single ILC1, ILC2 or ILC3 subset and therefore represent lineage-restricted ILCP, whereas the remainder are multi-potent ILCP that can give rise to 2 or more separate Lin⁻CD7⁺ ILC lineages (Figure 3E; Table S1). B cell and T cell potential was not observed. Second, within the multi-potent ILCP population, a substantial fraction (between 9-17%) are able to generate all three ILC subsets and likely represent immature uncommitted ILCP. Moreover, clonal IFN- γ^+ cultures also comprise EOMES⁺ NK cells demonstrating that some PB ILCP have the potential to generate both 'helper' and 'cytotoxic' ILC lineages at the single cell level. Third, a subset of Lin⁻CD7⁺ ILC clones failed to produce any cytokine tested (Figure 3E; Table S1). As these clones maintained high level of CD117 but lacked other ILC markers, they may represent ILCP that have not further differentiated (Figure S3B). Fourth, Notch signals clearly influence the cell fate potential of CD117⁺ ILCP as multi-potentiality and development of ILC3-containing cells was enhanced on OP9-DL4 (Figure 3E; Table S1). Together, these data identify PB CD117⁺ ILC as a circulating pool of partially committed ILC progenitors. The comparison of bulk and clonal assays clearly demonstrate the importance of the single cell approach to define heterogeneity of CD117⁺ ILC cell fate potential and to establish functional multipotency.

Circulating CD117⁺ *ILCP have multi-ILC potential in vivo in humanized mice*

We next assessed the *in vivo* potential of PB CD117⁺ ILCP. Severely immunodeficient mouse strains engrafted with human CD34⁺ hematopoietic stem cell (HSC) progenitors generate human lymphoid (B, T, NK) and myeloid (DC, macrophage, neutrophils) cell subsets (reviewed in (Shultz et al., 2012)). We used <u>BALB/c</u> <u>*Rag2^{-/-}Il2rg^{-/-}Sirpa*^{NOD} (BRGS)</sub> mice that are permissive for robust multi-lineage human hematopoietic cell engraftment (Legrand et al., 2011). Human PB CD34⁺ HSC and CD117⁺ ILCP were transferred to newborn BRGS mice; cytokine supplementation (human IL-2, -7, -1 β , -23) was provided and mice were analyzed 4 weeks later (Figure 4A). BRGS mice engrafted with human CD34⁺ HSC developed CD19⁺ B cells and CD14/CD33⁺ myeloid cells in the bone marrow, while CD3/CD5⁺ T cells and Lin⁻ CD7⁺ NK/ILC were detected in the gut (Figure 4B). In contrast,</u> BRGS mice receiving PB CD117⁺ ILCP developed Lin⁻CD7⁺ cells but no myeloid cells, B cells or T cells. Human CD45⁺ progeny from transferred CD117⁺ ILCP were detected in multiple organs, including the spleen, lung, gut and liver (Figure 4C). At each of these tissue sites, EOMES⁺ NK cells as well as diverse CD127⁺ ILC subsets could be identified that produced IFN- γ , IL-13, IL-17A and/or IL-22 *ex vivo* upon stimulation (Figure 4C). These results demonstrate that PB CD117⁺ cells comprise committed ILCP having the capacity to generate all known ILC subsets and NK cells *in vivo*.

Human CD117⁺ ILCP develop from CD34⁺ HSC in vivo

We next interrogated the developmental relationship between CD34⁺ HSC and CD117⁺ ILCP in 'humanized' BRGS mice (Figure 5A). As expected (Legrand et al., 2011), bone marrow, lung, liver and spleen harbored human CD45⁺ cells, including a variety of lineage⁺ T, B and myeloid cells (Figure 5B, data not shown). Moreover, within the subset of Lin⁻CD7⁺ cells, a clearly defined subpopulation of CD127⁺CD117⁺ cells could be discerned in multiple tissues that lacked T-BET and EOMES expression (Figure 5B and 5C). These included CD127⁺CD117⁺ cells that expressed low levels of GATA-3 and ROR γ t and were NKp44⁻ (Figure 5D) and therefore resembled PB CD117⁺ ILCP. *Ex vivo* stimulation failed to elicit cytokine production from CD127⁺CD117⁺ cells (Figure 5E). We sorted these cells and cultured them in the presence of IL-2, IL-7, IL-1 β and IL-23. Expanded cells contained subsets able to produce IFN- γ , IL-13, IL-17A and IL-22 (Figure 5E) thereby confirming the presence of human ILCP. These results demonstrate that fetal liver CD34⁺ HSC can give rise to CD117⁺ ILCP *in vivo*.

Human CD117⁺ ILCP are present in fetal liver, cord blood and adult lung

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We next assessed the stage of development when human CD117⁺ ILCP arise. Fetal liver (FL) harbors several immature hematopoietic precursor populations (Rollini et al., 2007) and is proposed as a site for the development of lymphoid tissue inducer cells in the mouse (Cherrier et al., 2012). Lin⁻CD127⁺ ILC within FL contain a predominant CD117⁺ subset (Figure S4A). Interestingly, these cells express RORyt at levels exceeding their peripheral blood counterparts (Figure 1C and S4A) and moreover express CCR6, Neuropilin-1 (NRP-1) but not NKp44 (Figure S4A and S4B). Despite these differences, FL CD117⁺ ILC did not produce significant amounts of IL-17A or IL-22 after stimulation (Figure S4C) suggesting that they were not fully mature ILC3. Nevertheless, when FL CD117⁺ ILC were expanded in vitro, IL-17A-producing ILC3 were abundantly generated. Moreover, IL17A⁺ ILC3 developed on stromal cells lacking the Notch ligand DL4 suggesting that Notch engagement is not necessary for this process (Figure 6A). Interestingly, bulk cultures of FL CD117⁺ ILC also contain IL-13- and IFN- γ -producing cells, although at lower frequency. Clonal analysis revealed that FL CD117⁺ ILC harbor a high proportion of ILC3 committed progenitors. Still, a substantial fraction of multi-potent ILCP can be revealed in the presence of Notch ligands (Figure 6B and 6C; Table S2). These results demonstrate that the human FL harbors CD34⁻ CD127⁺CD117⁺ multi-potent ILCP. The enrichment of ILC3-committed progenitors in this organ suggests that environmental signals may direct the further specification of multi-potent ILCP towards an ILC3 fate.

We next characterized CD117⁺ ILC from human CB. Like their PB counterparts, CB CD117⁺ ILC lacked NKp44, CCR6 and NRP-1 and were CD45RA⁺ (Figure S4A and S4B). Moreover, CB CD117⁺ ILC failed to express ROR γ t and T-BET but were GATA-3^{lo}, thus resembling PB ILCP (Figure 1C and S4A). Like PB CD117⁺ ILC, CB CD117⁺ ILC did not produce cytokines *ex vivo* after stimulation (Figure S4C). However, culture of CB CD117⁺ ILC generated IFN- γ^+ ILC1, IL-13⁺ ILC2 and IL-17A⁺ or IL-22⁺ ILC3 (Figure 6D). No T, B

or myeloid cells were detected in cultures of CB CD117⁺ ILC (data not shown). Further clonal analysis revealed that CB CD117⁺ ILC harbored a diverse mix of uni-potent and multi-potent ILCP (Figure 6E and 6F; Table S2). Unlike FL CD117⁺ ILC, CB CD117⁺ ILC were not biased towards ILC3-committed progenitors, but more closely resembled PB CD117⁺ ILCP. As for ILCP from PB or FL, Notch stimulation resulted in an enhanced frequency of multi-potent ILCP (especially those having the potential for IL-17A⁺ and IL-22⁺ ILC3) and reduced the frequency of cytokine⁻ ILC clones.

We also examined the phenotype and potential of CD117⁺ ILC from adult lung tissue. Lung CD117⁺ ILC harbored discreet populations of NKp44⁺ and RORγt⁺ ILC but were largely CD45A⁻ (Figure S4A and S4B). Bulk cultures of lung CD117⁺ ILC generated diverse cytokine-producing ILC subsets and EOMES⁺ NK cells (Figure 6G); further analysis using clonal assays defined the NKp44⁻ fraction of lung CD117⁺ ILC as a mixture of uni-potent and multi-potent ILCP (Figure 6H and 6I; Table S2). These results demonstrate that a variety of ILCP, including multi-potent progenitors, are present in human mucosal tissues.

ILC precursors reside within secondary lymphoid tissues

As human secondary lymphoid tissues (lymph nodes, tonsils) harbor diverse ILC subsets and their precursors, we further characterized tonsillar CD117⁺ ILCP and assessed their cell fate potential. CD117⁺ ILC from pediatric tonsils harbor a predominant NKp44⁺ ILC3 subset that can be stimulated to produce IL-17A and IL-22 (Hoorweg et al., 2012). This population also appears to have extensive functional plasticity as stimulation (using IL-1β, IL-12, IL-23) modifies their cytokine production (Bernink et al., 2015; Bernink et al., 2013; Cella et al., 2010). Within tonsillar CD117⁺ ILC, we found that NKp44⁻ cells were CD45RA⁺ and NRP-1⁻, while NKp44⁺ cells were CD45RA⁻ and NRP-1⁺ (Figure S4B). These findings suggest that NKp44⁺ ILC3 are more mature and differentiate from NKp44⁻ cells (Bernink et al., 2015).

al., 2015). However, cytokine production profiles were different in bulk cultures from tonsillar NKp44⁻ versus NKp44⁺ CD117⁺ ILC (Figure 6J and 6M). In particular, IFN- γ^+ cells and IL-13⁺ cells were more obvious in cultures derived from NKp44⁻ cells, especially on OP9 stroma (Figure 6J).

In order to better understand the relationship between NKp44⁻ and NKp44⁺ CD117⁺ ILC, we generated clones from both subsets and analyzed their cytokine-production potential. Striking differences were observed. Clones derived from NKp44⁺ CD117⁺ ILC were highly enriched ILC3 producing IL-17A and/or IL-22 (Figure 6N and 6O; Table S2). A fraction of clones co-expressed IFN-y (14%) that likely represent 'plastic' ILC3 that may up-regulate T-BET as previously shown (Bernink et al., 2015). In contrast, clones derived from NKp44-CD117⁺ ILC were quite heterogeneous with cells producing not only IL-22 and/or IL-17A but also abundant single IFN- γ^+ clones as well as single IL-13⁺ clones (Figure 6K and 6L; Table S2) that were not detected from NKp44⁺ CD117⁺ ILC (Figure 6N and 6O; Table S2). The presence of IFN- γ^+ ILC1 clones was unexpected given previous reports that tonsillar CD127⁺ ILC1 differentiate into IL-22 producing ILC3 in the presence of IL-2, IL-23 and IL-1 β (Bernink et al., 2015). Lastly, multi-potent ILCP giving rise to three ILC subsets were only found in NKp44⁻ CD117⁺ ILC. Taken together, these results suggest that tonsillar CD117⁺ ILC are quite heterogeneous comprising NKp44⁻ ILCP as well as NKp44⁺ ILC3. Furthermore, our extensive clonal assays clearly allow the definition of ILCP repertoires that is not visualized at the bulk culture level.

RORC-deficient patients harbor ILCP but fail to generate IL-17A⁺ ILC3

A committed ILCP in human secondary lymphoid tissue with a CD34⁺CD45RA⁺CD117⁺ phenotype was shown to highly express the TF RORC (Scoville et al., 2016). As CD117⁺ ILCP are developmentally downstream from CD34⁺ HSC (Figure 5), it

is possible that the previously described CD34⁺CD45RA⁺CD117⁺ ILCP subset is an intermediate in this pathway. In order to address whether RORC is required for generation of human CD117⁺ ILCP, we studied blood ILC in RORC-deficient patients. RORC deficiency in humans is associated with mycobacterial disease and mucocutaneous candidiasis and previous studies demonstrated that RORC is essential for differentiation of Th17 cells that protect against these pathogens (Okada et al., 2015). A reduction in the frequency of CD117expressing ILC was noted in patients with RORC deficiency, while CD56^{bright} and CD56^{dim} NK cells were unaffected (Figure 7A and 7B). In contrast, ILC1 were present and the percentage of ILC2 from total ILC was significantly increased in the absence of RORC (Figure 7B). Sorted CD117⁺ ILC from control and RORC-deficient patients were cultured as described above. Robust growth of Lin⁻CD7⁺ cells was observed with no significant difference between WT and RORC-deficient cells. We identified diverse cytokine producing cells in these cultures, including those producing IFN- γ , IL-13 or IL-22, however, there was a total absence of IL-17A-producing cells (Figure 7C). Development of EOMES⁺IFN- γ^+ NK cells was not affected by the absence of RORC. These results demonstrate that RORC is not required for the development of NK cells, ILC1, ILC2 or IL-22⁺ ILC3 but is essential for the generation of IL-17A⁺ ILC3 from ILCP in humans.

Discussion

In this report, we identify and characterize human ILC precursors (ILCP) as a subset of Lin⁻CD7⁺CD127⁺CD117⁺ cells in cord and adult blood as well as fetal liver and several adult tissues. Human ILCP give rise to all mature ILC subsets that are capable of producing a range of cytokines (IFN- γ , IL-13, IL-17A, IL-22) after *in vitro* culture in an appropriate cytokine environment or after transfer *in vivo* to immunodeficient mice. Human ILCP also generate EOMES⁺ NK cells demonstrating their potential for both cytokine-producing and cytotoxic ILCs. This is the first evidence for a circulating ILCP in any species and further demonstrate the broad systemic distribution of ILCP within human lymphoid and nonlymphoid tissues including mucosal sites (Figure S5).

The identification of human ILCP was possible thanks to a robust OP9 stromal cellbased assay that could assess ILC potential at the single cell level. With this approach, we analyzed over 1300 ILC clonal cultures and identified uni-potent ILCP that could give rise to IFN- γ^+ ILC1, IL-13⁺ ILC2 or IL-17A⁺ and/or IL-22⁺ ILC3 as well as and multi-potent human ILCP that could generate two or more ILC subsets. We demonstrate that human CD34⁺ HSC can develop *in vivo* into CD117⁺ cells that harbored ILCP with multi-lineage ILC potential. These results suggest a model for human ILCP development whereby pluripotent CD34⁺ HSC differentiate multi-potent ILCP progressively into (with the CD34-CD7⁺CD127⁺CD117⁺CD45RA⁺ phenotype) with potential for ILC1, ILC2, ILC3 and EOMES⁺ NK cells. Both CD34⁺ HSC and multi-potent ILCP are present in fetal liver suggesting that this tissue is permissive for this transition and previously described human tonsillar ILCP (Scoville et al., 2016) may represent an intermediate in this pathway. The absence of CD34⁺CD117⁺CD45RA⁺ ILC precursors in BM, as well as cord and adult blood (Scoville et al., 2016) suggests that these ILCP arise locally. The circulating and tissueresident human ILCP that we describe also harbor cells with more restricted uni-potent ILC. While we have not identified a marker that allows distinction between multi-potent and unipotent ILCP, we assume that they retain a precursor-product relationship.

Transcriptomic and epigenomic analysis of circulating human ILCP revealed a signature consistent with a partial specification to the ILC lineage. TF known to be critical for ILC development in mice (including *TCF7*, *TOX*, *ID2* and *GATA3*; (Klose et al., 2014; Seehus et al., 2015; Serafini et al., 2014; Yang et al., 2015)) were clearly up-regulated in ILCP compared to circulating HSC. In contrast, signatures of early B and T lymphopoiesis were not obvious, consistent with the inability of ILCP to generate adaptive lymphocytes *in vitro* or *in vivo*. ILC group-defining TFs (*BCL2, TBX21, EOMES, RORC*) were either absent or expressed at low levels suggesting commitment to ILC1, ILC2 or ILC3 was not yet completed. Interestingly, the loci encoding these factors were still 'poised' as evidenced by abundant H3K4Me2 epigenetic modifications. This chromatin landscape likely facilitates rapid generation of differentiated ILC subsets following cytokine-driven expansion (Lara-Astiaso et al., 2014; Zook et al., 2016).

The striking similarities between mature ILCs and fully differentiated T_H cells are well recognized (reviewed in (Spits et al., 2013)). T_H cells arise following antigen activation of naive T cells within specialized zones of secondary lymphoid tissues. While the analogous sites and structures that promote mature ILC differentiation are not known, it is interesting to consider circulating and tissue-resident ILCP as the functional innate counterpart of naive T cells. Both naïve T cells and ILCP are CD45RA⁺ and CD69⁻ suggestive of a resting state. Human and mouse ILCP clearly demonstrate the potential for further differentiation to all known ILC subsets and lack expression of the signature TF that drive mature ILC effector functions. As such, ILCP resemble naïve T cells that can differentiate to diverse T helper subsets under appropriate environmental signals. Nevertheless, human ILCP have signature TF in a poised state which contrasts with the situation in naïve T cells where these loci are actively repressed (Shih et al., 2016). Moreover, ILCP expand extensively in the presence of cytokines, whereas naïve T cell homeostasis is primarily maintained through cytokine-driven survival (Marrack and Kappler, 2004). Taken together, ILCP appear to have some properties in common with naïve T cells, although a number of important differences exist that are consistent with their designation as immature progenitors.

While uni-potent and multi-potent ILCP were identified in every human tissue sample tested, there were clearly differences in the relative proportions of ILCP that were uni- or multi-potent. It is therefore likely that each tissue harbors a unique ILCP 'repertoire' conditioned by environmental signals. One may include Notch signals that appear to influence ILCP multi-potency and ILC3 fate, particularly in the fetal liver. It is remarkable that other uni-potent ILCP were rarely detected in this organ, suggesting that at this stage of fetal development, the liver microenvironment may deliver signals that strongly polarize ILCP towards ILC3. These results corroborate earlier findings in the mouse (Cherrier et al., 2012). Notch-mediated signaling has been proposed to play a role in directing lymphoid cell fate decisions in the mouse fetal liver, promoting the development of T-lineage primed precursors as well as modifying ILCP homeostasis (Chea et al., 2016; Dallas et al., 2005). Soluble factors are also likely to be involved in ILCP 'repertoires' as these cells express several cytokine receptors (IL-1R, IL-2R, IL-18R) that allow them to sense tissue inflammation and stress.

Regulation of TF expression dictates ILC fate as well as function. Signature TF have been identified for ILC subsets that 'fix' their differentiation at the level of surface phenotype and effector outputs, especially for cytokines (reviewed in (Serafini et al., 2015)). The TF RORC helps define the ILC3 subset and is required for development and maintenance of ILC3 (but not ILC1, ILC2 or NK cells) in mice (Satoh-Takayama et al., 2008; Sawa et al., 2010). As expected, RORC is expressed by human ILC3 and in committed ILC3P (Montaldo et al., 2014). The recent report that all human ILC subsets express RORC (Scoville et al., 2016) suggested a broader role for this TF in human ILC differentiation. By analyzing blood from RORC null patients, we could show that RORC was not required for global ILC differentiation in humans, but rather was critical for IL-17 production by the ILC3 subset. ILCP in RORC-deficient patients retained the capacity to generate other cytokine-producing ILC and NK cell subsets. It is noteworthy that IL-22⁺ ILC3 developed in a RORC-independent fashion, suggesting compensatory pathways for these cells in humans.

We have previously shown that OP9 stroma minimizes human ILC2 plasticity (Lim et al., 2016) and extend this observation by showing that in this culture system the vast majority of NKp44⁺ ILC3 clones retain their functional attributes with little plasticity towards the ILC1 phenotype. Moreover, previous reports proposed that ILC1 clones rapidly differentiate towards an ILC3 fate in the presence of IL-1 β (Bernink et al., 2015), whereas we found that ILC1 clones on OP9 stroma (containing IL-1 β) retained their IFN- γ signature. As such, our culture system appears useful to assess signals that promote 'primary' ILC fate from ILCP.

Finally, our identification of circulating and tissue-resident human ILCP suggests a concept of 'ILC-poiesis on-demand' in which ILC differentiation can occur in any tissue and at any age. A recent study using parabiosis in mice has proposed that ILCs are long-lived tissue-resident cells that do not recirculate under steady-state and some inflammatory conditions (Gasteiger et al., 2015). In contrast, other reports have indicated that the half-life of several mucosal ILC subsets in the mouse is on the order of weeks, suggesting that these cells must be renewed (Sawa et al., 2010). The discovery of a circulating ILCP provides a mechanism to replenish tissue ILCs in response to steady-state losses and in the context of infection and inflammation.

Author Contributions

Conceptualization, A.I.L. and J.P.D.; Methodology, A.I.L., Y.L., R.S., F.P., H.S-M., H.Y.; Software, R.S., F.P.; Formal Analysis, A.I.L., S.L-L., R.S., F.P.; Investigation: A.I.L., Y.L., R.S., F.P., A.C., N.S., L.S., G.M-R.; Resources: A.P., L.L., R.C., D.T., P.G., L.A.M., L.R., J-M.S., R.H., T.G., J-L.C.; Writing, A.I.L.; Funding Acquisition, J.P.D.; Supervision, J.P.D.

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Conflict of Interest Statement

James Di Santo is a founder and stakeholder in AXENIS (Paris, France). The other authors declare no conflict of interest.
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Main Figure Legends

Figure 1. Characterization of peripheral blood CD117⁺ILC

(A) Gating strategy for FACS analysis of human PB ILC. Total ILC were gated on viable CD45⁺ Lin (CD3⁻ CD4⁻ CD5⁻ TCR $\alpha\beta$ ⁻ TCR $\gamma\delta$ ⁻ CD14⁻ CD19⁻) CD7⁺ CD127⁺ cells (red). NK cells are identified by CD56^{Dim} (grey), ILC2 are marked by CRTh2⁺ cells (green) and CD117⁺ ILC are gated on CRTh2⁻ CD117⁺ population (blue). (B) Percentage of total ILC from viable CD45⁺ and CD117⁺ ILC from total ILC of healthy adult donors in PB. Results from 27 healthy individuals (Median). (C) Expression of surface phenotypes (NKp44, IL1R1 and CD69) and intracellular transcription factors (EOMES, T-BET, GATA-3 and ROR γ t) of PB CD117⁺ ILC and gut CD117⁺NKp44^{+/-} ILC. (D) Functional profiles (IFN- γ , IL-13, IL-22 and IL-17A) of PB CD117⁺ ILC and gut CD117⁺NKp44^{+/-} ILC in response to 3 h PMA/iono stimulation. Data representative of at least 3 individuals analyzed from at least 3 independent experiments. See also Figure S1.

Figure 2. The transcriptional signature and chromatin landscape of CD117⁺ ILC

(A) Schematic for RNA-Seq and ChIPm-Seq analyses. (B) Heatmap depicting normalized ChIPm-Seq signal showed H3K4me2 intensity of 36449 high-confidence regulatory region, of which 1989 were unique to CD117⁺ ILC. Venn diagram shows the overlap between three categories of H3KMe2⁺ regions ('CD34⁺ HSC-specific'; 'CD117⁺ ILC-specific' and 'shared'). (C) Heatmap of significantly enriched molecular pathways associated with genes near GREs uniquely identified in CD117⁺ ILC (blue) or CD34⁺ HSC (orange). (D) Heatmap depicting k-means clustering of differentially expressed genes between CD117⁺ ILC (blue) and CD34⁺ HSC (orange) as detected by RNA-Seq. (E) Overlap of the 1540 genes most highly expressed by CD117⁺ ILC and 2283 genes associated with 'CD117⁺ ILC-specific'.

H3KMe2⁺ GREs. Selected genes essential for ILC development are highlighted. (F) RNA-seq reads and corresponding H3K4Me2 traces are shown for selected loci. See also Figure S2.

Figure 3. Cloning reveal multi-ILC lineage potential of CD117⁺ ILC in vitro

(A) FACS analysis of bulk cultured CD117⁺ ILC for surface phenotypes, intracellular EOMES and cytokine expression after 3h PMA/ionomycin stimulation to identify NK cells (EOMES⁺ cells), ILC1 (IFN- γ^+ cells), ILC2 (IL-13⁺) and ILC3 (IL-22⁺ and/or IL-17A⁺). (B) Expansion of bulk cultured CD117⁺ ILC (10 days) in stromal cell-free conditions with cytokines (20 ng/ml each). Results from four independent donors; ns, *p*>0.05; ******, *p*<0.01; ********, *p*<0.0001 using paired Student's t test compared to IL-2, -7 only condition (Median). (C) Schematic diagram and morphology of CD117⁺ ILC-OP9 stromal co-culture system. (D) Single PB CD117⁺ ILC were cultured on OP9 or OP9-DL4 stromal cells for 14-18 days. Cells were stimulated with PMA/iono 3h before cytokine analysis. IL-17A and IL-22 producing cells were analyzed after gating on IFN- γ ⁻ IL-13⁻ cells. Positive clones were considered when at least 100 viable human CD45⁺ cells were detected by FACS. Presence of an ILC subset was scored when more than 5% of corresponding cytokine was detected in total viable CD45 cells. (E) Pie chart depicting all possible ILC combinations detected. Frequency of each single or multi ILC differentiation among total positive wells. Data summarized from four independent experiments with one donor each. See also Figure S3.

Figure 4. CD117⁺ ILC effectively give rise to multi-ILC lineage in vivo

(A) Schematic diagram of *in vivo* transfer experiment. (B-C) Newborn BRGS mice were inhjected with PB CD117⁺ ILC or CD34⁺ HSC and analyzed 4 weeks later. (B) FACS analysis for lymphocytes and myeloid surface markers gated on viable human CD45⁺ cells from bone marrow and gut. (C) ILC analysis within viable human CD45⁺ cell using CD127,

NKp44 and CD117 expression, intracellular EOMES and intracellular cytokines (IFN- γ , IL-13, IL-22 and IL-17A) production in lung, gut, liver and spleen of BRGS mice transferred with CD117⁺ ILC. Representative data of at least 4 mice in each group from 3 independent experiments.

Figure 5. Human ILCP accumulate in human immune system (HIS) mice

(A) Schematic diagram of generation of HIS mice. Fetal liver CD34⁺CD38⁻ HSC were intrahepatically injected into newborn BRGS mice. Mice were analyzed 8 to 9 weeks later. (B) Representative FACS analysis of human ILCP (Lin⁻ CD7⁺ CD127⁺ CD117⁺) in spleen, BM, lung and liver of HIS mice. (C) Percentage of ILCP from total human CD45⁺ in spleen, BM, lung and liver of HIS mice. (Median). (D) FACS analysis of surface phenotypes and transcription factors profiles of ILCP and NK cells from spleen of HIS mice. (E) Cytokine production of splenic CD117⁺ ILC pre- and post-culture on OP9-DL4 with IL-2, -7, -1 β , and -23 for 10 days. Cytokine production was analyzed after 3 h of PMA/iono stimulation. Representative data of 8 mice from at least 3 independent experiments.

Figure 6. *In vitro* bulk and clonal assay of CD117⁺ ILC from lymphoid and non-lymphoid organs.

Bulk (100-300 cells) or single CD117⁺ NKp44^{+/-} CD117⁺ ILC from different organs were FACS sorted into 96-well round bottom plate pre-seeded with OP9 or OP9-DL4 and supplied with IL-2, -7, -1 β and -23 (20 ng/ml each). Intracellular cytokine production in respond to 3h PMA/iono stimulation was performed to identify ILC1 (IFN- γ^+), ILC2 (IL-13⁺) and ILC3 (IL-22⁺ and/or IL-17A⁺) after 8-10 days bulk culture (A, D, G, J, M) or 14-18 days single cell culture (B-C, E-F, H-I, K-L, N-O). Representative FACS analysis of bulk cultured CD117⁺ NKp44⁺ ILC isolated from (A) FL, (D) CB, (G) lung, (J) tonsil and CD117⁺ NKp44⁺ from (M)

tonsil. Pie chart depicting all possible ILC combinations after clonal expansion of CD117⁺ NKp44⁻ ILC from (B-C) FL, (E-F) CB, (H-I) lung, (K-L) tonsil and (M-N) CD117⁺ NKp44⁺ from tonsil on OP9 and OP9-DL4. See also Figure 3 legend. Data summarized from at least 2 independent experiments with one donor each. See also Figure S4

Figure 7. Development potential of ILCP from RORC^{-/-} patients

(A) FACS analysis of peripheral blood ILC subsets from healthy and RORC^{-/-} patient samples. (B) Percentage of NK^{Dim} and NK^{Br} from viable CD45⁺ cells, ILC1, ILC2 and ILCP from total ILC of PB of healthy and RORC^{-/-} patients. Result from 22 healthy donors and 2 RORC^{-/-} patients. ns, p>0.05; *, p<0.05; **, p<0.01 using unpaired Student's t test (Median) (C) ILCP from healthy donor or RORC^{-/-} patients were FACS sorted and cultured on OP9-DL4 with IL-2, -7, -1 β and -23 for 8 days. Surface phenotypes, intracellular EOMES expression and cytokine production profiles were analyzed.

Supplementary Figure Legends

Figure S1. CD117⁺ ILC gating strategy, Related to Figure 1

(A) FACS analysis of ILCs from human peripheral blood and gut. CD117⁺ ILC were gated on lymphoid size, singlet and viable CD45⁺ Lin⁻ CD7⁺ CD127⁺ CRTh2⁻ CD117⁺ population. (B) Surface phenotypes of blood CD117⁺ ILC (blue) and NK cells (grey).

Figure S2. Transcriptional and epigenetics signature of PB CD117⁺ ILC, Related to Figure 2

(A) Annotation of H3K4Me2⁺ regions as defined by their overlap with known genomic features. (B) Comparative transcriptional and epigenetics profiles of CD117⁺ ILC and CD34⁺ HSC for selective loci. Bar graphs show gene expression values identified by RNA-Seq from CD117⁺ ILC (blue) and CD34⁺ HSC (orange) (n=2, error bars denote SEM). Adjacent genome browser screenshots display normalized H3K4Me2 ChIPm-Seq signal in CD117⁺ ILC (blue) or CD34⁺ HSC (orange). Arrowheads mark regions of CD117⁺ ILC-specific H3K4Me2 enrichment.

Figure S3. Cytokines environment dictate progeny of CD117⁺ ILC, Related to Figure 3

(A, B) 100-300 CD117⁺ ILC were expanded in stromal cell-free condition using different cytokines combination (20 ng/ml each) for 10 days. Representative FACS analysis for cytokines (IFN-γ, IL-13, IL-22 and IL-17A) production in response to 3h PMA/iono stimulation. (B) Representative FACS analysis for surface (CD7, CD117, CD94), intracellular TF (EOMES), and cytokine production (IFN-γ, IL-13, IL-22 and IL-17A) production in respond to 3h PMA/iono stimulation of cytokine⁻ clones.

Figure S4. Gating strategy and characteristic of CD117⁺ ILC from fetal liver, cord blood, lung and tonsil, Related to Figure 6

(A) Gating strategy for FACS analysis to identify CD117⁺ ILC from fetal liver, cord blood, lung and tonsil. Surface expression for CRTh2 and NKp44, intracellular TF (RORγt, GATA-3 and T-BET) was compared between CD117⁺ NKp44⁻ ILC (solid blue), CD117⁺ NKp44⁺ (dashed blue), CRTh2⁺ ILC2 (green) and CD56⁺ NK cells (grey). (B) Functional profiles of CD117⁺ ILC (blue), and CD56⁺ NK cells (grey) from FL, CB, lung and tonsil in response to 3 h PMA/iono stimulation. (C) Surface phenotypes of CD117⁺NKp44⁻ ILC (solid blue), CD117⁺NKp44⁺ (dashed blue), CRTh2⁺ ILC2 (green) and CD56⁺ NK cells (grey) from FL, CB, lung and Tonsil. (D) Table summarize number of clones detected for all possible ILC combinations from CD117⁺ NKp44⁻ isolated from FL, CB, lung, tonsil or CD117⁺ NKp44⁺ from tonsil 14-18 days co-culture with OP9 or OP9-DL4.

Figure S5. Model for Human 'ILC-poiesis'

Human ILCP (Lineage⁻CD127⁺CD117⁺CD45RA⁺) within fetal and adult tissues as well as in the circulation can be detected that have potential for all ILC subsets and NK cells.

Supplementary Table Legends

Table S1. Number of clones detected for all possible ILC combination from 340 CD117⁺ ILC clones co-culture with OP9 or OP9-DL4 stroma cells, Related to Figure 3E

Table S2. Number of clones detected for all possible ILC combination from CD117⁺ NKp44^{+/-} ILC clones from different tissues co-culture with OP9 or OP9-DL4 stroma cells, Related to Figure 6

STAR Methods

Contact for reagent and resources sharing

Further information may be directed to the corresponding author (james.di-santo@pasteur.fr).

Experimental model and subject details

Human blood and tissues samples

Blood samples from healthy donors were obtained from Establissement Français du Sang (EFS, Paris). Blood samples from patients with *RORC* mutation have been previously reported (Okada et al., 2015). Fetal liver with gestational age ranging from 14 to 20 weeks were obtained from Advanced Bioscience Resources Inc. following approval of an institutional medical ethical committee at Institut Pasteur and the French Ministry of Education and Research (IF-20080451). Umbilical cord blood was collected from normal deliveries. Tonsils were obtained from pediatric patients undergoing routine tonsillectomy (Scientific Institute for Research and Health Care "Casa Sollievo della Sofferenza"). Lung and colon samples were obtained from patients undergoing therapeutic resection (Dr. X. Norel and J.-M. Sallenave, Hôpital Bichat; Dr. M Allez, Hôpital Saint Louis). Informed consent was obtained from all patients and included protocols approved by the institutional review boards of Necker Medical School, Paris Descartes University, Hôpital Bichat, Hôpital Saint Louis, Assistance Publique - Hôpital de Paris.

In vivo analysis using human immune system (HIS) mice model

<u>B</u>ALB/c <u>Rag2^{-/-}Il2rg^{-/-}Sirpa^{NOD}</u> (BRGS) mice have been described (Legrand et al., 2011) and were maintained in isolators at Institut Pasteur. CD34⁺ HSC or CD117⁺ ILC were sorted from peripheral blood of healthy donors using a FACS Aria. Fetal liver CD34⁺ HSC were isolated using CD34 Microbead Kit (Miltenyi). For *in vivo* transfer experiment, 1-3 x 10⁵ CD117⁺ ILC or CD34⁺ HSC were intrahepatically injected into sublethal irradiated (3Gy)

new born (3-7 days-old) BRGS mice together with 0.3 μ g of IL-2 and -7 (Miltenyi). Mice received IL-2, -7, -1 β , -23, -25 and -33 (0.3 μ g each) by intraperitoneal injection weekly and were analyzed four weeks post-transplantation. For generation of HIS mice, 2 x 10⁵ fetal liver-derived CD34⁺ CD38⁻ HSC were intrahepatically injected into sublethal irradiated (3Gy) new born (3-7 days-old) BRGS mice. Mice were sacrificed 8-9 weeks post-injection. Experiments were approved by ethical committee at Institut Pasteur and validated by French Ministry of Education and Research (MENESR#02162.02).

Method details

Cell isolation from blood, tonsil, gut, fetal liver and lung

Human peripheral blood mononuclear cells (PBMC) from CB and PB were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation. Single cell suspension from fetal liver and tonsil was achieved by mechanical disruption through 70-µm filters. Lung and intestine samples were minced and digested with Liberase TL (25 µg/ml; Roche) and DNase I (50 µg/ml; Sigma-Aldrich) for 45min in 37°C shaking incubator. Digested tissues were passed through 70-µm filters. Lymphocytes from liver, lung and gut were isolated by Ficoll-Paque density gradient centrifugation.

FACS analysis and cell sorting

For FACS analysis, cells were first stained with Flexible Viability Dye eFluor 506 (eBioscience) for 10 min following by 20 min surface antibodies staining with Brilliant Stained Buffer (BD) on ice. For experiment involving intracellular TF staining, cells were fixed, permeabilized and stained using Foxp3/Transcription Factor Staining Buffer Kit (eBioscience). For intracellular cytokines staining, cells were stimulated with PMA (10ng/ml; Sigma) plus Ionomycin (1 μ g/ml; Sigma) in the presence of Golgi Plug (BD) for 3 h in 37°C

incubator. Cells were fixed, permeabilized and stained by Cytofix/Cytoperm Kit (BD). Samples were acquired on LSRFortessa (BD) and analyzed by FlowJ10 (Tree Star).

For cell sorting from healthy PB, PBMC were first depleted of T cell, B cell, pDC and monocytes by labeling with biotin-conjugated anti-CD3, anti-CD4, anti-CD19, anti-CD14, anti-CD123 followed by anti-biotin microbeads (Miltenyi) according to manufacturer's instructions. Sorting from CB and tissues were performed without lineage depletion. Bulk populations were sorted to a purity \geq 99% or as single cell index sorting (both using FACSAria II; BD).

Bulk RNA isolation, library construction, sequencing and analysis

10³ cells from each population were FACS sorted directly into 50 μl of lysis/binding buffer (Life Technologies). mRNA was captured with 15 μl Dynabeads oligo(dT)(Life Technologies), washed according to manufacturers' instructions and eluted at 85°C with 6 μl of 10mM Tris-HCl (pH7.5). We used a derivation of MARS-Seq as described (Jaitin et al., 2014), developed for single-cell RNA-seq to produce expression libraries of two replicates per population. Libraries were sequenced at an average depth of 5 million reads per library on the Ilumina NextSeq and aligned to the human reference genome (hg19). Reads were mapped using hisat (version 0.1.6); duplicate reads were filtered if they had identical UMIs. Expression levels were calculated and normalized to the total number of reads using HOMER software (http://homer.salk.edu).

Chromatin immunoprecipitation and sequencing (Chip-Seq) using ChipMentation

FACS sorted cells (20-50K) were immediately crosslinked in PBS containing 1% formaldehyde (Sigma) for 10 min at room temperature for ChIP-Seq analysis. Crosslinking was quenched by adding glycine (0.125M final concentration) followed by 5 min incubation at room temperature. Cells were placed on ice, washed with PBS and snap-frozen for storage

at -80°C. Pellets were processed in parallel to minimize technical variation. Cells were resuspended in 100µl sonication buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH8 and 1x EDTA-free complete protease inhibitors; Roche) and transferred to a 0.65ml Bioruptor sonication tube (Diagenode). After 15 min incubation on ice, cells were sonicated for 30 cycles (30 sec ON - 30 sec OFF) using a Bioruptor Pico sonicator (Diagenode) to shear chromatin down to ± 250 bp fragments. Chromatin was equilibrated by adding 900µl 10x ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8, 167mM NaCl) and incubated overnight at 4°C with 1µl of H3K4Me2-specific antibody (ab32356, Abcam) or normal rabbit IgG as a negative control (sc-2027, Santa Cruz). In addition, 20µl of protein A Dynabeads (Thermo Fisher Scientific) per IP were blocked in PBS containing 0.1% BSA (Sigma) by incubation overnight at 4°C. The next day, beads were resuspended in the original volume with ChIP dilution buffer and added to the chromatin extracts. After 2 hours of incubation at 4°C, beads were collected and washed with Low Salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 150mM NaCl), High Salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 500mM NaCl) and LiCl buffer (10mM Tris-HCl pH8, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 0.5% deoxycholic acid). Chromatin-antibody immobilized on magnetic beads were then subjected to tagmentation as recently described (Schmidl et al., 2015). Eluted DNA was purified using MinElute spin columns (Qiagen) and amplified for 8-12 cycles using Nextera PCR primers. Libraries were purified using dual (0.5x-2.0x) SPRI Ampure XP beads (Beckman Coulter), pooled (up to 10 per sequencing run) and sequenced on a NextSeq500 (Illumina) running a single-read 75bp protocol.

ChIP-Seq data processing, analysis and visualization

Reads were demultiplexed using BaseSpace (Illumina) and aligned to the mouse genome (mm10 build) using Bowtie (Langmead and Salzberg, 2012) with standard settings, removing

reads that could not be uniquely mapped. Indexed and sorted bam files were parsed to HOMER (Heinz et al., 2010) for further analysis. Tag directories were generated for each sample with removal of duplicate reads (-tbp 1 option). BedGraph files displaying normalized counts (reads per million) were generated for direct visualization in the UCSC Genome Browser (https://genome.ucsc.edu/) using the makeUCSCfile HOMER script. H3K4Me2 enriched regions were identified using HOMER findPeaks with -region -size 1000 -minDist 2500 options. Overlapping and non-overlapping regions between two samples were identified using the intersect function of BEDTools (Quinlan and Hall, 2010) or the HOMER mergePeaks script (-d given option) requiring a minimal overlap of 1bp. Sets of cell type-specific H3K4Me2+ regions were visualized as heatmaps with Java TreeView (Saldanha, 2004). Regions/peaks were assigned to putative target genes GREAT (McLean et al., 2010). GREAT was subsequently used to calculate enrichments of these genes for known pathway signatures using the whole genome as background.

Bulk and single cell culture

All *in vitro* culture experiments were performed in Yssel's medium (18432890) supplemented with 2% human AB serum (EFS). 2-3 x 10^3 stromal cells were pre-seeded in 96-well round bottom plates one night before culture. For bulk culture, 100-300 FACS sorted cell were plated on the stromal cells. For cloning experiment, cells were index-sorted directly into the 96-well plates pre-seeded with stromal cells. Cytokines IL-2, -7 (20 ng/ml each, Miltenyi), IL-12, -18, -25, -33, -1 β , -23 (20 ng/ml each, R&D) were provided in various combinations as indicated. For bulk culture, fresh cytokines and medium were replenish every 5 days and analyzed after 10 days expansion. For cloning experiment, cytokines and medium were replenished every 7 days and analyzed after 14-18 days of culture.

Quantification and statistical analysis

Data are represented as Median unless specified. The sample size for each experiment and the replicate number of experiments are included in the figure legends.

Data and software availability

Data resources

The accession number for the RNA-Seq and ChIPm-Seq analysis have been deposited in the NCBI Gene Expression Omnibus under accession numbers GEO: GSE90834 and GSE90640.

Additional resources

Yssel's Medium

Yssel's Medium is prepared in house by using IMDM (Invitrogen) plus 0.25 (w/v) bovine serum albumin (Sigma), 1.8 ug/L 2-amino ethanol, 40 ug/L Apo-transferrin, 5 ug/L insulin and penicillin/Streptomycin.























None							
ILC1							
ILC2							
ILC3							
Multi-potent							
ILC1,2							
ILC1,3							
ILC2,3							
ILC1,2,3							

0

0

Figure 4









Figure 7



	OP9				OP9-DL4					
	Exp1	Exp2	Exp3	Exp4	Total	Exp1	Ex2	Exp3	Exp4	Total
Non	0	0	12	7	19	0	0	1	0	1
ILC1	2	2	16	21	41	0	3	16	7	26
ILC2	22	2	20	10	54	12	0	6	5	23
ILC3	1	0	2	1	4	0	3	5	3	11
ILC1,2	7	1	13	16	37	5	0	1	8	14
ILC1,3	0	0	2	2	4	3	13	8	9	33
ILC2,3	2	2	3	6	13	3	5	5	5	18
ILC1,2,3	0	5	7	5	17	7	6	6	6	25
No. Clones	34	12	75	68	189	30	30	48	43	151
Multi-potent (%)	26	66	33	42	38	60	80	46	65	60

Table S1

Table S2

Tissues	Fetal	Liver	Cord	Blood	Lu	ng	Tonsil			
Subset	CD117 [⁺] NKp44 ⁻								CD117 ⁺ NKp44 ⁺	
Feeder	OP9	DL4	OP9	DL4	OP9	DL4	OP9	DL4	OP9	DL4
None	0	0	32	9	3	0	36	6	8	1
ILC1	0	2	38	14	42	11	29	19	3	1
ILC2	2	0	13	11	14	0	19	22	0	0
ILC3	15	74	6	1	16	9	6	6	33	58
ILC1,2	1	3	12	7	18	1	13	4	0	0
ILC1,3	5	66	5	25	17	46	6	12	19	11
ILC2,3	2	28	3	5	4	9	7	3	4	6
ILC1,2,3	2	17	12	11	12	8	16	10	0	0
No. Clones	27	190	121	83	126	84	132	78	67	77
Multi-potent (%)	37	60	27	58	41	76	32	36	34	22













PAPER III:

IN VIVO EFFICACY OF UMBILICAL CORD BLOOD STEM CELL DERIVED **NK** CELLS IN THE TREATMENT OF METASTATIC COLORECTAL CANCER

Colorectal cancer (CRC) is the fourth leading cause of cancer related deaths in the world. Despite substantial advances in the treatment of metastatic CRC (mCRC) over the last decades that have contributed to better survival rates, the disease is still frequently fatal. Monoclonal antibodies targeting the Epidermal Growth Factor Receptor (EGFR) pathway are approved for the treatment of patients with advanced CRC either in combination with chemotherapy or, as monotherapy, in chemo-refractory conditions. However, mutations in tumor suppressor genes and proto-oncogenes in EGFR signaling pathways, such as in RAS, BRAF and PIK3CA are common in patients with CRC. These mutations represent a poor prognostic marker and render anti-EFGR mAbs ineffective, leaving 42% of the chemo-refractory mCRC population without standard treatment option.

Besides the blockade of the EGFR-ligand interaction on tumor cells, therapeutic mAbs can also interact with NK cells triggering antibody-dependent cell-mediated cytotoxicity (ADCC). Several studies have shown a dysfunctional phenotype and poor infiltration of NK cells in the CRC tissue from early stages on, together with an immunosuppressive tumor microenvironment. Among the different strategies to increase NK cell-mediate anti-tumor response, autologous NK cell infusion has been evaluated in several solid tumors with unsuccessful results.

In this study, we used BRGS mice to evaluate the therapeutic potential of a novel combinatorial treatment in an EGFR-expressing RAS-mutant colorectal carcinoma model. This treatment consisted on umbilical cord blood stem cell derived NK cells and the monoclonal antibody cetuximab. The anti-tumor efficacy of UCB-NK cells against cetuximab resistant human EGFR⁺RAS^{mut} colon cancer cells was observed both *in vitro* and *in vivo* with UCB-NK showing enhanced anti-tumor cytotoxicity against colon cancer independent of EGFR and RAS status.

In vivo efficacy of umbilical cord blood stem cell derived NK cells in the treatment of metastatic colorectal cancer

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Key words: EGFR, RAS mutation, cetuximab, metastatic colorectal cancer, A-PBNK, UCB-NK, allogeneic NK cell immunotherapy.

Abstract:

Therapeutic monoclonal antibodies (mAbs) against the epidermal growth factor receptor (EGFR) act by inhibiting EGFR downstream signaling and by eliciting an NK cellmediated anti-tumor response. The IgG1 mAb cetuximab has been used for treatment of RAS^{wt} metastatic colorectal cancer (mCRC) patients, showing limited efficacy. In the present study we address the potential of adoptive NK cell therapy to overcome these limitations investigating two allogeneic NK cell products, i.e. activated peripheral blood NK cells (A-PBNK) and umbilical cord blood stem cell derived NK cells (UCB-NK). While cetuximab monotherapy was not effective against EGFR⁻ RAS^{wt}, EGFR⁺ RAS^{mut} and EGFR⁺ BRAF^{mut} cells, A-PBNK were able to initiate lysis of EGFR⁺ colon cancer cells irrespective of RAS or BRAF status. Cytotoxic effects of A-PBNK (but not UCB-NK) were further potentiated significantly by coating EGFR⁺ colon cancer cells with cetuximab. Of note, a significantly higher cytotoxicity was induced by UCB-NK in EGFR-RAS^{wt} ($42 \pm 8\%$ versus $67 \pm 7\%$), EGFR+ RAS^{mut} (20 \pm 2% versus 37 \pm 6%) and EGFR⁺BRAF^{mut} (23 \pm 3% versus 43 \pm 7%) colon cancer cells compared to A-PBNK and equaled the cytotoxic efficacy of the combination of A-PBNK and cetuximab. The anti-tumor efficacy of UCB-NK cells against cetuximab resistant human EGFR⁺RAS^{mut} colon cancer cells was further confirmed in an in vivo preclinical mouse model where UCB-NK showed enhanced anti-tumor cytotoxicity against colon cancer independent of EGFR and RAS status. As UCB-NK cells have been proven safe in a recently conducted phase I clinical trial in acute myeloid leukemia, a fast translation into clinical proof of concept for mCRC could be considered.

Introduction:

Colorectal cancer (CRC) is the fourth leading cause of cancer related deaths in the world(1). Despite substantial advances in the treatment of metastatic CRC (mCRC) over the last decades that have contributed to better survival rates(2, 3), the disease is still frequently fatal. Monoclonal antibodies targeting the Epidermal Growth Factor Receptor (EGFR) pathway, such as panitumumab and cetuximab are approved for the treatment of patients with advanced CRC either in combination with chemotherapy or, as monotherapy, in chemo-refractory conditions (4). Cetuximab (CET) and panitumumab block the interaction between EGFR and its ligands, thus inhibiting the downstream RAS-signalling cascade and tyrosine kinase activation(5). However, mutations in tumour suppressor genes and proto-oncogenes in EGFR signalling pathways, such as in RAS, BRAF and PIK3CA are common in patients with CRC. These mutations represent a poor prognostic marker and render anti-EFGR mAbs ineffective, leaving 42% of the chemo-refractory mCRC population without standard treatment option(6, 7).

Besides the blockade of the EGFR-ligand interaction on tumor cells, therapeutic mAbs can also interact with Natural Killer (NK) cells triggering antibody-dependent cellmediated cytotoxicity (ADCC)(8-10), and this can translate into superior anti-tumor effects(11). Two NK cell subsets can be identified based on the expression of CD16, the low affinity FcTwo NK receptor. The majority of NK cells are CD56^{dim}CD16⁺, and play an active role in NK cell cytotoxicity and are capable of performing ADCC upon IgG1 engagement via CD16, whereas CD56^{bright}CD16⁻ NK cells are mainly immune regulatory in function, secreting cytokines, and are less cytotoxic than CD56^{dim} cells(12). NK cell functions are tightly regulated by a delicate balance between activating receptors (like the natural cytotoxicity receptors NKp46, NKp30 and NKp44, or C-type lectin-like receptor NKG2D)(13) and Major Histocompatibility Complex (MHC) class I binding inhibitory receptors, including Killer-cell immunoglobulin-like receptors (KIRs), LIR1/ILT2 and NKG2A/CD94(14). The importance of NK cells in controlling tumors has been extensively demonstrated since their identification 40 years ago (15-17).

Several studies have shown a dysfunctional phenotype and poor infiltration of NK cells in the CRC tissue from early stages on, together with an immunosuppressive tumor microenvironment(18, 19). Hence, various strategies e.g. using cytokines or therapeutic ADCC enhancing mAbs, have been explored to increase NK cell numbers and function and to enhance their trafficking to tumor sites(20). Another approach entails the adoptive transfer of *in vitro* manipulated and expanded autologous or allogeneic NK cells. Autologous NK cells so far have failed to demonstrate significant therapeutic benefits in solid tumors (21-23). Therefore, the focus has shifted to the development of allogeneic NK cells as a potential adoptive cell therapy for treatment in solid tumors. Previously, we demonstrated that the combination of allogeneic activated PBNK (A-PBNK) cells and CET can effectively target RAS mutant (RAS^{mut}) CRC tumors(24).

Here, we compared two feeder cell free allogeneic NK cell products, i.e. activated peripheral blood NK cells (A-PBNK) and cord blood stem cell derived NK cells (UCB-NK), alone or in combination with cetuximab for anti-tumor effects against RAS^{mut}CRC.

Materials and Methods

Cell lines

Cell lines A431 (epidermoid carcinoma), COLO320, SW480 and HT-29 (colon carcinoma) were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified medium (DMEM; Invitrogen, Carlsbad CA, USA) containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands). Cell cultures were passaged every 5 days and maintained in a 37°C, 95% humidity, 5% CO2 incubator.

PBNK isolation and activation

Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized blood of healthy donors (6 males, 4 females, age range = 56-64 and CRC patients (8 males, 2 females, age range = 66-74) after written informed consent and according to protocols approved by the institutional review board of VU University Medical Center, Amsterdam (NCT01792934). Blood samples were collected at baseline and after the first cycle of firstline palliative chemotherapy consisting of oral capecitabine (1000 mg/m², bid, day 1-14), i.v. oxaliplatin (130 mg/m², day 1) and i.v. bevacizumab (7.5 mg/kg, day 1, in 4/10 mCRC patients). PBMC were isolated using Lymphoprep[™] (STEMCELL Technologies, Cologne, Germany) density gradient centrifugation. CD56⁺ NK cells were isolated from PBMC using a MACS Human NK cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. PBNK cells purity and viability were checked using CD3 VioBlue, CD56 APC Vio 770, and CD16 APC (Miltenyi Biotech) and 7AAD (Sigma Aldrich, Zwijndrecht, The Netherlands). Isolated PBNK cells were activated overnight with 1000U/ml IL-2 (Proleukin®; Chiron, München, Germany) and 10 ng/ml IL-15 (CellGenix) for use in cytotoxicity assays. The parameters compared before and after stimulation with cytokines were NK purity ($87 \pm 5\%$ versus $84 \pm 2\%$), NK CD16⁺, $92 \pm 12\%$ versus $88 \pm 8\%$) and NK viability ($89 \pm 5\%$ versus $84 \pm 8\%$), respectively.

Flow cytometry

The antibody staining mix for the assessment of NK cell functionality consisted of CD45 VioGreen, CD14 VioBlue, CD19 VioBlue and SYTOX® Blue, together with CD3 PerCP-Vio 700 and TCR- γ δ PerCP-Vio700 to exclude dead cells, debris and non-NK populations from PBMC. NK cells were identified by the expression of CD45⁺CD3⁻CD56⁺ cells, and further characterized for NK functionality by plotting against CD16 APC, CD25 VioBrightFITC, CD107a PE, and NKp44 PE-Vio770 and for NK cell phenotype by plotting against NKG2A PE-Vio770, NKG2C PE, NKG2D PerCP-Cy5.5 and PanKIR2D FITC. All

antibodies were supplied by Miltenyi Biotec except SYTOX® Blue (Thermo Fisher Scientific, Berlin, Germany).

UCB-NK cultures

Allogeneic NK cells (UCB-NK) were generated from cryopreserved umbilical cord blood (UCB) hematopoietic stem cells as previously described(25). CD34⁺ UCB cells from six UCB-donors were plated (4x10⁵/ml) into 12-well tissue culture plates (Corning Incorporated, Corning, NY, USA) in Glycostem Basal Growth Medium (GBGM®) (Clear Cell Technologies, Beernem, Belgium) supplemented with 10% human serum (HS; Sanquin Bloodbank, Amsterdam, The Netherlands), 25ng/mL of SCF, Flt-3L, TPO, and IL-7 (CellGenix, Freiburg, Germany). In the expansion phase II, from day 9 to 14, TPO was replaced with 20ng/mL IL-15 (CellGenix). During the first 14 days of culture, low molecular weight heparin (LMWH) (Clivarin®; Abbott, Wiesbaden, Germany) in a final concentration of 20 μ g/ml and a low-dose cytokine cocktail consisting of 10 pg/ml GM-CSF (Neupogen), 250 pg/ml G-CSF and 50 pg/ml IL-6 (CellGenix) were added to the expansion cultures. Cells were refreshed with new medium twice a week and maintained at 37°C, 5% CO². On day 14, the NK cell differentiation process was initiated by addition of NK cell differentiation medium consisting of the same basal medium with 2% HS but with high-dose cytokine cocktail consisting of 20ng/ml of IL-7, SCF, IL-15 (CellGenix) and 1000 U/ml IL-2 (Proleukin®; Chiron, München, Germany). Cultures were refreshed every 2-3 days and maintained till day 42. Five UCB-NK cultures were used for cytotoxicity assays and one UCB-NK culture for in vivo studies (both with a CD56⁺ cell purity of >95%). UCB-NK CD16 levels in matured UCB-NK cells were monitored using an antibody mix of human CD45VioGreen (1:11), CD56 APC-Vio770 l(1:11) and CD16 APC (1:11). Similarly, UCB-NK CD16 expression in BRGS mice was monitored using an antibody mix of BV650 antimouse CD45 (clone 30-F11), Alexa Fluor® 700 anti-human CD45 (clone HI30), PE-CF594
anti-human CD56 (clone B159), all from BD and APC-Vio770 anti-human CD56 (clone REA196) and APC CD16 (clone REA423) both from Miltenyi Biotec.

NK cell cytotoxicity assays

Flow cytometry was used for the read-out of cytotoxicity assays. Target cells (COLO320, SW480 and HT-29 were labelled with $5 \,\mu$ M pacific blue succinimidyl ester (PBSE; Molecular Probes Europe, Leiden, The Netherlands) at a concentration of 1×10^7 cells per ml for 10 min at 37°C. The reaction was terminated by adding an equal volume of FCS, followed by incubation at room temperature for 5 min after which stained cells were washed twice and suspended in DMEM + 10% FCS to a final concentration of 5 x 10^{5} /ml. Overnight activated PBNK cells and UCB-NK cells were washed with PBS and suspended in Glycostem Basal Growth Medium (GBGM) + 2% FCS to a final concentration of 5 x 10^{5} /ml. Target cells were co-cultured with effector cells at an E:T ratio of 1:1 in a total volume of 250 μ 1 in 96well flat-bottom plates (5 x 10^4 targets in 100 μ l of DMEM + 10% FCS incubated with 5 x 10^4 effectors in 100 μ l of GBGM + 2% FCS, further supplemented with 25 μ l of GBGM + 2% FCS and DMEM + 10% FCS medium). NK cells and target cells alone were plated out in triplicate as negative controls. Target cells were coated with 5 μ g/ml cetuximab (Merck, Darmstadt, Germany) for 1h at 4°C. To measure degranulation of NK cells, anti-CD107a PE (Miltenyi Biotech) was added in 1:20 dilution at the beginning of the assay. After incubation for 4hr at 37°C, cells were harvested and stained with CD56 APC Vio 770 (1:25) and CD16 APC (1:25) (Miltenyi Biotech) and 7AAD (1:500) (Sigma Aldrich). Degranulation of NK cells was measured by detecting cell surface expression of CD107a.

In vivo studies

The EGFR⁺RAS^{mut} SW480 cell line and EGFR⁺⁺⁺RAS^{wt} A431 cell line were stably transduced with Gaussia Luciferase (Gluc) for in vivo studies. Lentiviral (LV) supernatant of Cerulean Fluorescent Protein (CFP) positive Gluc virus (LV-CFP-Gluc) was kindly provided by Dr. Tom Würdinger(26). SW480 and A431 cells with Gluc expression of 95% were used for mouse studies.

Immunodeficient BRGS mice (BALB/c*Rag2^{tm1Fwa}Il2rg^{tm1Cgn}Sirpa^{NOD}*) were used in this study. Twenty-four adult mice (male, 8 weeks old) received an intravenous (i.v) tail vein injection with 0.5 x 10⁶ SW480 Gluc cells at day 0 and were randomized into 4 groups. Group A only received SW480 cells, group B received SW480 in combination with cetuximab intraperitoneally (i.p., 0.5 mg, days 1, 4, 7), group C received SW480 in combination with UCB-NK i.v. (1x10⁷, days 1, 4, 7), and group D received SW480 cells in combination with UCB-NK i.v. (1x10⁷, days 1, 4, 7) and cetuximab i.p. (0.5 mg, days 1, 4, 7). Groups C and D received i.p. 0.5 μ g IL-15 + 7.5 μ g IL-15R α every 2-3 days from day 0 till day 14. Further, three adult mice received i.v tail vein injection of 0.5 x 10⁶ A431 Gluc cells at day 0 and were treated with 0.5mg cetuximab (i.p., 0.5mg days 1, 4, 7), was used as a cetuximab efficacy control. Treatment effects were monitored using blood Gluc levels and bioluminescence imaging (BLI). All manipulations of BRGS mice were performed under laminar flow conditions.

Blood Gluc quantification in vitro

Secreted Gluc was measured according to a protocol described previously(27). Ten μ l of blood was collected by capillarity into EDTA containing Microvette® CB tubes. Blood samples were distributed in 96 well black plates then mixed with 100 μ l of 100mM Gluc substrate native coelenterazine in PBS (P.J.K. GmbH; Kleinblittersdorf, Germany) and 5 minutes later light emission was quantified. Blood that was withdrawn before tumor inoculation served to determine a baseline value. Measurements were done twice a week until day 35. Gluc activity was measured using IVIS spectrum luminescence detector (PerkinElmer, Villebon-sur-Yvette, France). Data obtained were quantified using Living Image 4.0 software (PerkinElmer, Villebon-sur-Yvette, France).

Bioluminescence imaging in vivo

Mice were anesthetized using isofluorane gas in an induction chamber at a gas flow of 2.5 pm. Retro orbital injection of coelenterazine (4mg/kg body weight) was administered and mice were placed in the anaesthesia manifold inside the imaging chamber and imaged within 5 mins following substrate injection. Mice were placed into the light chamber and overlay images were collected for a period of 15min using IVIS spectrum in vivo imaging system (PerkinElmer, Villebon-sur-Yvette, France). Images were then analysed using Living Image 4.0 software (PerkinElmer, Villebon-sur-Yvette, France).

Ethics statement

Animals were housed in isolators under pathogen-free conditions with humane care and anaesthesia was performed using inhalational isoflurane anaesthesia to minimize suffering. Experiments were approved by the Institut Pasteur's ethical committee for animal use in research, Comité d'étique en expérimentation animale (CETEA) #89, protocol reference # 2007–006 and validated by the French Ministry of Education and Research (Reference # 02162.01).

Statistical analysis

Data were analyzed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Differences between conditions were determined using one-way ANOVA or two-way ANOVA with multiple comparisons between column means, unpaired-t-test and log rank (Mantel-cox) test as deemed appropriate. A p-value of <0.05 was considered statistically significant.

Results:

Highly dysfunctional NK cells in CRC patients

Flow cytometry was used to determine the frequency, phenotype and functionality of NK cells in PBMC of healthy volunteers (n=10, age range 56-64, 6 males/4 females) and patients with metastatic CRC (n=10, age range 66-74, 8 males/2 females) before and after the first cycle of first line palliative chemotherapy consisting of oral capecitabine (1000 mg/m2, bid, day 1-14), i.v. oxaliplatin (130 mg/m2, day 1) and i.v. bevacizumab (7.5 mg/kg, day 1, in 4/10 mCRC patients). As illustrated in figure 1A, mCRC patients harbored on average a 20% lower percentage of CD3⁻CD56⁺NK cells in the total CD45⁺ lymphocyte population as compared to healthy controls (p<0.05). These lower NK rates, which are in line with a previous report in colorectal cancer(28), further declined after the first cycle of chemotherapy (p<0.01).

We next evaluated whether this quantitative NK cell defect was also accompanied by functional defects in the NK cell population. For this purpose, the ability of NK cells from healthy volunteers and mCRC patients to induce both natural cytotoxicity and mediate ADCC of the epidermoid carcinoma cell line A431 (MHC-Ilow, EGFRhigh, KRAS^{wt}) was assessed. For ADCC tumor target cells were coated with cetuximab before the addition of NK cells. It was evident that the cytotoxic potential of NK cells from mCRC patients, as reflected by degranulation (i.e. CD107a surface expression), was highly impaired both before chemotherapy and after the first cycle of chemotherapy. Though NK cells of mCRC patients were capable of ADCC, as evidenced by significant increases in degranulation when target cells were coated with cetuximab (p<0.05), levels were still low compared to those observed in healthy volunteers. (Figure 1B). Of note, although the NK cells of healthy volunteers and mCRC patients expressed similar levels of CD16 (Figure 1C), this did not translate into comparable levels of ADCC. NKp44 expression, known to reflect the activation status of NK cells, was similar between the HD and mCRC groups used in NK cytotoxicity experiments (Figure 1D). Furthermore, no significant differences were observed in expression levels of NK activating (NKG2D, NKG2C) and NK inhibiting (NKG2A, KIR2D) receptors between healthy controls and CRC patients (Supplementary figure 1).

Enhanced in vitro cytotoxicity of colon cancer cells mediated by UCB-NK cells

In order to explore novel therapies to replace dysfunctional NK cells in patients with advanced CRC, we tested two different sources of allogeneic NK cell products (A-PBNK and UCB-NK), that could eventually be used for adoptive transfer strategies. We next compared the activity of A-PBNK cells (age range 22-37 years) and UCB-NK cells using a flow based NK cell cytotoxicity assay based on detection of 7-AAD accumulation in tumor cells. Three different cell lines of colon cancer origin were compared, i.e. COLO320 (EGFR- RAS^{wt}), SW480 (EGFR⁺ RAS^{mut}) and HT-29 (EGFR⁺ RAS^{wt}, BRAF^{mut}). As expected, addition of cetuximab to EGFR- RAS^{wt} COLO320 cells did not result in increased killing. Of interest, lysis was consistently and significantly higher (p<0.01) using UCB-NK compared to A-PBNK. As reported previously, the combination of cetuximab and A-PBNK resulted in increased killing of EGFR⁺RAS^{mut} SW480 and EGFR⁺ BRAF^{mut} HT-29 via ADCC(24). CD16 was expressed by $88 \pm 8\%$ (n=5) of A-PBNK after overnight stimulation with cytokines and by $7 \pm 2\%$ (n=5) of UCB-NK cells at the end of the 35-day culture period. No added effect of cetuximab was observed when using UCB-NK cells, which is possibly related to their lower in vitro CD16 levels(29). Of note, tumor cell lysis induced by UCB-NK cells was comparable to that observed with the combination of A-PBNK and cetuximab (Figure 2A). Measurements of NK cell degranulation reflected equivalent trends observed for tumor cell lysis (Figure 2B). These results show that UCB-NK cells have superior cytotoxic efficacy over A-PBNK cells against cetuximab resistant colon cancer cells in vitro.

UCB-NK cells inhibit in vivo tumor growth and increase survival

To address whether UCB-NK cells exhibit similar anti-tumor efficacy in vivo, we transferred Gluc transduced SW480 cells to immunodeficient mice (BRGS; see methods). SW480 cells are EGFR⁺RAS^{mut} and cetuximab monotherapy resistant. Mice were divided into 4 groups of 6 mice per group: SW480 only (group A), SW480 + cetuximab (group B),

SW480 + UCB-NK (group C) and SW480 + UCB-NK + cetuximab (group D). Gaussia luciferase activity in whole blood was measured every three days to monitor the tumor burden (Supplementary figure 2). These data confirmed our in vitro observations that SW480 cells were resistant to cetuximab mediated growth inhibition (blue line). Of note, while treatment with UCB-NK cells alone significantly decreased the tumor load (green line), this effect was not increased by combining UCB-NK cells with cetuximab and thereby further confirmed both the inefficacy of cetuximab in treating RAS mutated tumors as well as the inability of cetuximab to induce ADCC of UCB-NK cells in vivo (orange line) (Figure 3). CD16 expression levels on UCB-NK cells were monitored in two mice upon adoptive transfer and increased from 6.0% before transfer to 14.0% (mouse 1) and 19.1% (mouse 2) at day 5 post UCB-NK cell infusion (data not shown).

While the blood Gluc assay measurements provided evidence of a reduction in the total tumor burden after UCB-NK treatment, we wanted to explore the impact of the therapy on the localization and size of the metastases. For that purpose, BLI was performed at day 35 after tumor inoculation. Figure 4A depicts four representative BLI images from each group at day 35 post-tumor injection and average radiance from range of interest (ROI) measurements are shown in Figure 4B. Mice from groups A and B showed a higher and more diffuse tumor load compared to mice treated with UCB-NK alone or in combination with cetuximab. To demonstrate the possibility of antitumor efficacy of cetuximab in the BRGS mouse model we performed a similar tumor challenge using the cetuximab sensitive A431 cell line, which bears wild-type RAS and overexpresses EGFR. A significant decrease in tumor load was observed when A431 tumors were treated with the same concentration of cetuximab as in the SW480 study (Figure 4C), confirming the in vivo functionality of cetuximab. We next assessed whether treatment of SW480 bearing mice with UCB-NK cells alone or in combination with cetuximab translated into a survival advantage (Figure 5). Indeed, treatment of mice with UCB-NK cells alone resulted in a significant prolongation in their life span (p=0.01), whereas combinatorial therapy did not add significantly to this. Treatment with cetuximab alone did not translate into a significant survival advantage, consistent with the observed effects on tumor growth.

Discussion

To test the cytotoxic potential of NK cells for treating advanced colorectal cancer patients, we compared their functional status before and after chemotherapy. We observed that peripheral blood NK cell numbers were reduced in mCRC patients and that residual NK cells were dysfunctional and unable to mount a strong effector response when stimulated with an NK cell sensitive tumor target. Though an increase in NK cell cytotoxicity was observed when tumor target cells were coated with the anti-EGFR mAb cetuximab, reflecting a capacity for ADCC, cytotoxicity was still significantly lower (both before and after chemotherapy) than that observed in healthy controls. These data indicate a decreased functional state of NK cells in patients with mCRC, which is in line with studies in mice where the cytokine production and anti-tumor activity of adoptively transferred NK cells were highly affected following long-term exposure to tumors(30). Through recognition of MHC class I molecules KIRs prevent NK cells from targeting healthy cells while allowing them to detect tumor or infected cells with low or downregulated expression of MHC class I in a process known as "missing self"(31). Severely diminished or aberrant expression of MHC class I has been reported in the majority of colorectal adenocarcinomas(32),(33), which makes them an ideal target for NK cell-mediated killing. Although NK cells are infrequent in colorectal tissues(18), several independent studies investigated the clinical impact of NK cell infiltration on the prognosis of CRC, as well as in other types of carcinoma. These clinical studies, including a recent tissue microarray of 1414 CRC biopsies, led to the conclusion that NK cell infiltration in tumors correlated with better overall response rates and progressionfree survival in CRC patients(34-37), suggesting that therapies aimed at boosting NK cell functions could be beneficial in mCRC and possibly also in other types of cancer.

We evaluated and compared the cytotoxic efficacy of two different sources of feeder cell free allogeneic NK cells, i.e. A-PBNK cells and in vitro expanded and differentiated

UCB-NK cells. In vitro NK cell cytotoxicity experiments revealed that the cytotoxic activity of UCB-NK cells against CRC cells was significantly higher than that of A-PBNK cells and in addition demonstrated that, while an increase in cytotoxicity through ADCC was not evident with UCB-NK cells, their cytotoxic potential was still comparable to that observed with A-PBNK potentiated by cetuximab mediated ADCC. It is possible that the stronger cytotoxic effects of UCB-NK cells are due to the intense stimulation with cytokines in comparison to A-PBNK cells. The failure to observe ADCC-enhanced cytotoxicity with UCB-NK cells in vitro can be explained by their low expression levels of CD16(29). As we previously observed in vivo up-regulation of CD16 on UCB-NK cells upon their transfer to NOD/scid IL2Rg^{null} (NSG) mice(38), we decided to also test the efficacy of cetuximab treatment in combination with UCB-NK cells in an in vivo model. Treatment of SW480 RAS^{mut} tumors in BRGS mice with UCB-NK cells, resulted in control of disease progression and translated into a significantly longer survival. As expected, cetuximab monotherapy did not result in a decreased SW480 tumor load or improvement in survival, recapitulating the clinical data from patients bearing RAS^{mut} CRC tumors. Unexpectedly, we failed to demonstrate superior in vivo anti-tumor effects or survival when we combined the transfer of UCB-NK cells with cetuximab infusions. The underlying causes for this latter finding remain obscure but may be related to sub-optimal in vivo upregulation of CD16 in the used mouse model or CD16 polymorphisms in the employed batch of UCB-NK cells, both of which could have hampered efficient ADCC.

Taken together, UCB-NK cells displayed significant anti-tumor efficacy, suggesting a potential beneficial role for UCB-NK cells in the treatment of RAS and BRAF mutant CRC. As an important present limitation in treating mCRC patients is related to resistance to anti-EGFR mAbs, adoptive transfer of cytolytic UCB-NK cells could thus constitute a viable treatment option. Our in vitro and in vivo data demonstrating that adoptive transfer of UCB-NK cells alone was as effective as the combination of A-PBNK and cetuximab raises the possibility that UCB-NK administration could obviate the use of cetuximab in RAS^{wt} mCRC.

Furthermore, UCB-NK can also lyse RAS^{mut}CRC cells at levels higher than those observed with A-PBNK. Importantly, allogeneic NK cells have demonstrated their safety in clinical trials in several solid tumors(39, 40), and more specifically, the UCB-NK cell product used in our experiments was found to be safe in a clinical trial in Acute Myeloid Leukemia (AML) patients (Dolstra et al., 2016 manuscript submitted).

Several features make UCB-NK attractive for further clinical development. For example, our GMP based expansion and differentiation protocol reproducibly resulted in a more than 10,000-fold expansion of cytotoxic UCB-NK cells from single donors. Furthermore, UCB-NK cells can be supplied as an "off the shelf" product, stored in large aliquots facilitating multiple infusions. Also, the low immunogenicity by UCB grafts prevents adverse reactions that are prevalent after repeated PBNK transfusions(41). In this respect, it is relevant to mention that while NK cells in general are often inhibited by recognition of MHC class I molecules on the surface of tumor cells, UCB-NK display relatively low levels of Killer cell - immunoglobulin like receptors (KIRs) supporting their ability to effectively lyse MHC class I expressing tumor cells(29). Finally, the ability of UCB-NK cells to proliferate and home to liver, lungs, spleen and bone-marrow after adoptive transfer has been previously demonstrated in NSG mice (38), though additional studies are required to determine whether UCB-NK cells have a similar migratory pattern upon adoptive transfer in solid tumor patients. Together, these features and observations provide UCB-NK cells with several unique advantages for further development as a universal NK cell platform.

Considering the size and heterogeneity of the tumor mass in advanced stages of CRC and other types of cancer, UCB-NK may not provide a sufficient therapeutic effect as a single agent. However, rational combinations of UCB-NK cells with existing drugs or drugs that are in clinical development can be envisioned to further increase their efficacy. Previous studies have pointed out that the proteasome inhibitor (bortezomib)(42) and the immunomodulatory drug (lenalidomide)(43) sensitize tumor cells to NK mediated killing. In addition, UCB-NK cell application together with bispecific or trispecific antibodies by binding to UCB-NK cell

activating receptors can also increase NK cell tumor specificity(44). Though we did not specifically assess ADCC induced by other mAbs, it is very likely that the failure of UCB-NK to mediate ADCC is a more general phenomenon as this generally depends on binding to CD16/FcγRIII, which was found to be expressed at only low levels in the UCB-NK cell product. However, recent data from a clinical phase 1 study with the same UCB-NK cell product in patients with AML revealed significant upregulation of CD16 on UCB-NK cells post transfusion suggesting that the UCB-NK cell product may acquire the capacity to mediate ADCC in patients following adoptive transfer (Dolstra et al., manuscript submitted). Further, this phenomenon may also provide a strong rationale for combining UCB-NK cells with bispecific or trispecific killer cell engagers(45). Taken together, these approaches can substantially increase UCB-NK cell responses to advanced solid tumors, including mCRC.

In conclusion, in this study we have demonstrated the in vitro efficacy of UCB-NK cells against multiple colorectal cancer cell lines independent of EGFR expression and EGFR downstream signaling mutations, and in addition have demonstrated the in vivo antitumor efficacy of adoptively transferred UCB-NK cells against EGFR⁺RAS^{mut} tumors. As the adoptive transfer of UCB-NK cells (oNKord®) has been shown to be safe in patients with AML (CCMO nr NL31699 & Dutch trial register no 2818), our data provide a rationale for the clinical exploration of UCB-NK cells in the treatment of mCRC.

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Author contributions

Conceived and designed the experiments: JV SLL JPD TDG HVV JS HV. Performed the experiments: JV SLL NK FB. Analyzed the data: JV and SLL. Contributed reagents/materials/analysis tools: DH. Wrote the paper: JV and SLL.

Conflict of interest disclosures

JV, JS, NK and FB are employees of Glycostem Therapeutics; DH serves on the scientific advisory boards of Amgen and Pfizer. JPD is a stakeholder and founder of Axenis, SAS (France). The authors declare no conflict of interest.

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Results

(n=10)



• Healthy donors CRC baseline + CRC post chemo • Healthy donors + CET CRC baseline + CET CRC post chemo + CET



(A) Frequency of NK cells within PBMC from healthy controls and from mCRC patients at baseline and after the first cycle of chemotherapy. (B) NK cell degranulation in healthy controls and mCRC patients after a 4 hr co-culture of resting NK cells with A431 cells in the presence (open symbols) or absence (closed symbols) of cetuximab at an E:T ratio of 1:1. (C) Expression levels of resting NK cell CD16 and (D) NKp44 in healthy controls and in mCRC patients before and after 1 cycle of chemotherapy. Data represent mean \pm SEM from 10 mCRC patients and 10 age and sex matched healthy controls. *P < 0.05, **P < 0.01, ***P < 0.005, calculated with one-way ANOVA, multiple comparison between column means.



Figure 2: Ex vivo cytotoxic efficacy of A-PBNK and UCB-NK cells against CRC cells

(A) CRC cell lines COLO320 (EGFR⁻, RAS^{wt}), SW480 (EGFR⁺, RAS^{mut}) and HT-29 (EGFR⁺, RAS^{wt}, BRAF^{mut}) were subjected to NK killing using two allogeneic NK cell products, i.e. A-PBNK and UCB-NK cells. 7AAD (A) and CD107a (B) were measured after a 4 hr co-culture of A-PBNK and UCB-NK cells with CRC targets in the presence or absence of cetuximab at an E:T ratio of 1:1. Experiments were carried out in triplicate. Bars represent mean \pm SEM, n=5. *P < 0.05 and **P < 0.01, calculated with two-way ANOVA, multiple comparison between column means.



Figure 3: Significant anti-tumor effects of UCB-NK cells in vivo

Real time monitoring of tumor progression and treatment response was performed measuring Gluc levels from mice blood twice a week. Baseline Gluc values were obtained from all mice a day before tumor injection (day-1), and further monitoring continued until day 35. Blood Gluc levels were compared between control SW480 only (A) group and treatment groups SW480 + cetuximab (B), SW480 + UCB-NK (C) and SW480 + UCB-NK + cetuximab (D) for statistical significance. Data presented is from 6 mice per group (n=6). Scatter plots represent mean \pm SEM. *P < 0.05, calculated with unpaired-t test.



Figure 4: Successful tumor elimination by UCB-NK cells as revealed by bioluminescence imaging in vivo

(A) Four mice from control and treatment groups were imaged at day 35 for tumor load and distribution. Mice were injected retro-orbitally with Gluc substrate coelenterazine and images were acquired for 5 min. In SW480 control and SW480+cetuximab groups, tumor growth was extensive and highly disseminated, spreading to most parts of the body. However, in UCB-NK and UCB-NK + cetuximab groups there was a significantly lower tumor load, which was further verified by calculating the average radiance between groups as shown in figure B (n=4 mice per group). (C) Cetuximab functionality against EGFR⁺⁺⁺ RAS^{wt} A431 cells was tested in parallel to SW480 studies in BRGS mice (n=3 mice per group). For figures B and C, bars represent mean ± SEM. *P< 0.05 for figure B and figure C was calculated with unpaired t test.



Figure 5: Significant survival benefit in cetuximab resistant RAS mutant tumor bearing mice treated with UCB-NK cells

Kaplan -Meier survival curves were plotted for the total experimental study period from day 0 until day 65. Survival rates of SW480 (EGFR⁺, RAS^{mut}) tumor bearing mice (n=6 per group) following treatment with PBS only (black line), cetuximab only (blue line), UCB-NK only (green line) and UCB-NK + cetuximab (orange line) were plotted over time to monitor treatment outcome. Statistical differences between groups were calculated using log rank (Mantel-Cox) test and indicated in the figure.



Supplementary figure 1: Expression profiles of NK cell receptors in CRC patients. Resting NK cells within PBMC populations from healthy controls and from mCRC patients at baseline and after one cycle of chemotherapy were monitored for the NK cell activating receptors NKG2D (A), NKG2C (B) and NK cell inhibitory receptors NKG2A (C) and KIR2D (D). Data represent mean ± SEM from 10 mCRC patients and 10 healthy controls. Statistical difference was calculated with one-way ANOVA, multiple comparisons between column means.



🌢 Blood Gluc collection 🛐 Tumor bioluminescence imaging

Supplementary figure 2: Schedule of in vivo BRGS mouse experiments. BRGS mice were divided in 4 groups of 6 mice each. SW480 (A) is the control group, followed by treatment groups SW480+cetuximab (B), SW480+UCB-NK (C) and SW480+cetuximab (D). $0.5x10^6$ Gluc transduced SW480 cells per mouse were administered i.v. to all groups at day 0. On day 1 (dose I) post tumor injection, groups B and D were treated with 0.5mg of cetuximab i.p. and groups C and D were infused iv. with $10x10^6$ UCB-NK cells. Same doses of cetuximab and UCB-NK cells were again administered at day 4 (dose II) and day 7 (dose III) to the respective groups. 0.5μ g IL-15R α and administered to the UCB-NK-treated groups on days 1, 4, 7, 10 and 14. Treatment effects were monitored using blood Gluc levels and BLI *in vivo* imaging.

PAPER IV:

A NANOBODY BASED BISPECIFIC TARGETING APPROACH TO LEVERAGE THE POTENT AND WIDELY APPLICABLE TUMOR CYTOLYTIC CAPACITY OF MONOMORPHIC VΓ9VΔ2-T CELLS

We discussed earlier in this manuscript the unique features that make $V\gamma9V\delta2$ -T cells of major interest to cancer immunotherapeutic approaches. Indeed, clinical trials have been initiated to evaluate the use of $V\gamma9V\delta2$ -T cells in the treatment of both hematological and solid malignancies. Clinically explored approaches have included adoptive transfer of *ex vivo* expanded $V\gamma9V\delta2$ -T cells and the *in vivo* activation of $V\gamma9V\delta2$ -T cells through the administration of aminobisphosphonates NBPs or synthetic non-peptidic phosphoantigens (pAg), alone or in combination with low-dose IL-2. These $V\gamma9V\delta2$ -T cell-based therapeutic approaches were well tolerated and capable of inducing clinically relevant anti-tumor responses in several cases, however, the overall results were inconsistent, possibly related to the fact that these approaches induced a systemic $V\gamma9V\delta2$ -T cell activation without necessarily effecting their preferential accumulation in the tumor microenvironment, where these cells would be expected to exert their antitumor effects.

A recently developed approach to improve the activation and accumulation of $V\gamma 9V\delta 2$ -T cells in tumors are the nanobodies (or VHHs). These are formed by the variable antigen-binding region derived from heavy chain only antibodies, naturally occurring in camelids.

In this study we described the generation of a bispecific VHH construct that combines inhibition of the EGFR with the target-specific activation of effector $V\gamma9V\delta2$ -T cells using an agonistic anti- $V\gamma9V\delta2$ -TCR VHH. A BRGS model of colorectal carcinoma was used to evaluate *in vivo* the efficacy of this therapeutic strategy. $V\gamma9V\delta2$ -T cells activated in this manner produced pro-inflammatory cytokines such as IFN- γ and TNF- α *in vitro* and efficiently lysed EGFR expressing tumor both *in vitro* and *in vivo* regardless of *KRAS* mutations. This anti-tumor activity led to an overall lower tumor burden and improved survival compared to non-treated mice, confirming the therapeutic potential of VHHs in combination with $V\gamma9V\delta2$ -T cells.

A bispecific nanobody approach to leverage the potent and widely applicable tumor cytolytic capacity of $V\gamma 9V\delta 2$ -T cells

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One Sentence Summary: A novel bispecific nanobody-based construct targeting both $V\gamma9V\delta2$ -T cells and EGFR induces potent $V\gamma9V\delta2$ -T cell activation and subsequent tumor cell lysis both *in vitro* and in an *in vivo* mouse xenograft model, independent of *KRAS* and *BRAF* tumor mutation status and common $V\gamma9V\delta2$ -T cell receptor sequence variations.

Abstract

Though V γ 9V δ 2-T cells constitute only a small fraction of the total T cell population in human peripheral blood, they play a vital role in tumor defense and are therefore of major interest to explore for cancer immunotherapy. V γ 9V δ 2-T cell-based cancer immunotherapeutic approaches developed so far have been generally well tolerated and were able to induce significant clinical responses. However, overall results were inconsistent, possibly due to the fact that these strategies induced systemic activation of V γ 9V δ 2-T cells without preferential accumulation and targeted activation in the tumor. Here we show that a novel bispecific nanobody-based construct targeting both V γ 9V δ 2-T cells and EGFR induced potent V γ 9V δ 2-T cell activation and subsequent tumor cell lysis both *in vitro* and in an *in vivo* mouse xenograft model. Tumor cell lysis was independent of *KRAS* and *BRAF* tumor mutation status and common V γ 9V δ 2-T cell receptor sequence variations. In combination with the conserved monomorphic nature of the V γ 9V δ 2-TCR and the facile replacement of the tumor-specific nanobody, this immunotherapeutic approach can be applied to a large group of cancer patients.

Introduction

Although the majority of human T cells expresses an $\alpha\beta$ -TCR, a smaller proportion of T cells expresses a $\gamma\delta$ -TCR. The most predominant $\gamma\delta$ -T cell subset in human peripheral blood consists of V γ 9V δ 2-T cells that account for approximately 1-5% of all T cells. V γ 9V δ 2-T cells are able to induce apoptosis in a broad spectrum of cancer cells and their reduced frequency and/ or impaired functionality in the peripheral blood is a commonly observed phenomenon in cancer patients.(*1–8*) In melanoma patients, reduced V γ 9V δ 2-T cell levels in the tumor microenvironment are related to more advanced clinical stages and reduced V γ 9V δ 2-T cell levels in the peripheral blood were recently shown to be a negative predictor for response upon treatment with ipilimumab.(*9*, *10*) These observations clearly point to a vital role for V γ 9V δ 2-T cells in natural and induced immunity to cancer. In contrast to conventional T cells, ligand recognition by V γ 9V δ 2-T cells is independent of MHCmolecule presentation, tumor neo-epitope burden and classical immuno-editing.(*11*, *12*) This underscores their great potential as anti-tumor effector cells, a potential that has been hitherto largely untapped.

 $V\gamma 9V\delta 2$ -T cells become activated by the recognition of non-peptidic phosphoantigens (pAg).(13-15) These are upregulated by stressed cells, including malignant cells, as a consequence of an enhanced activity of the mevalonate pathway(16) or through the nonmevalonate pathway upon bacterial infection.(14, 17, 18) Furthermore, therapeutic agents such as aminobisphosphonates (NBP) can inhibit the mevalonate pathway and thus lead to intracellular pAg accumulation. Upon elevated intracellular levels of pAg in target cells, the GTPase RhoB translocates from the nucleus to the cytoplasm where it binds to the membrane protein butyrophilin 3A1 (BTN3A1, also known as CD277). This binding induces a conformational change of BTN3A1 that is sensed by the V γ 9V δ 2-T cell receptor (TCR) and results in rapid Vy9V δ 2-T cell activation.(19–23) Vy9V δ 2-T cells can be further activated by interactions between the NKG2D receptor expressed on most V γ 9V δ 2-T cells and by stress-related MICA, MICB and ULBP molecules that are upregulated in infected or transformed cells.(3, 24) This, in combination with enhanced pAg levels, allows V γ 9V δ 2-T cells to distinguish "normal" cells from "altered-self" or tumor cells.(25) Activated Vγ9Vδ2-T cells produce pro-inflammatory cytokines (e.g. IFN- γ , TNF- α and the chemokines MIP-1 and RANTES) in addition to cytolytic mediators (perforin, granzyme B) to induce the specific lysis of target cells, which is regulated through the perforin pathway or through Fasinduced apoptosis.(25, 26)

Their monomorphic recognition of activating ligands, their effective induction of tumor cell lysis and their rapid effector response provide $V\gamma 9V\delta 2$ -T cells with a unique combination of features that make them of major interest for cancer immunotherapeutic approaches. As a result, several clinical trials have been initiated to evaluate the use of $V\gamma 9V\delta 2$ -T cells in the treatment of both hematological and solid malignancies. Clinically explored approaches have included adoptive transfer of *ex vivo* expanded $V\gamma 9V\delta 2$ -T cells and the *in vivo* activation of $V\gamma 9V\delta 2$ -T cells through the administration of NBPs or synthetic pAg, alone or in combination with low-dose IL-2 treatment.(*27, 28*) These $V\gamma 9V\delta 2$ -T cellbased therapeutic approaches were well tolerated and capable of inducing clinically relevant anti-tumor responses in several cases. However, the overall results were inconsistent, possibly related to the fact that these approaches induced a systemic $V\gamma 9V\delta 2$ -T cell activation without necessarily affecting their preferential accumulation and activation in the tumor microenvironment, where these cells should exert their anti-tumor effects.

To date, various bispecific T cell engagers (BiTEs) targeting both CD3 and a tumor antigen through the coupling of single-chain variable fragments (scFv) have been developed and were shown to induce clinical responses.(29) However, as CD3 is expressed by all T cells, including immunosuppressive regulatory T cells (Tregs) that actually predominate in the tumor microenvironment and are related to poor prognosis(30), antibody-based constructs designed to exclusively trigger immune cells with a pro-inflammatory function, such as $V\gamma 9V\delta 2$ -T cells, might well constitute a more effective approach.(31) Recently, we have reported on the generation of a set of $V\gamma 9V\delta 2$ -TCR specific nanobodies with activating properties that could form the basis for a novel therapeutic approach aimed at tumor-specific $V\gamma 9V\delta 2$ -T cell accumulation and activation.(32) Nanobodies (or VHHs) are defined by the variable antigen binding regions derived from heavy chain only antibodies, naturally occurring in camelids (i.e. llamas, camels and dromedaries).(33, 34) Single-domain VHH have several advantages over full-length antibodies or scFv when used for the generation of multivalent and/or multispecific molecules. Due to the absence of light chain domains, pairing issues do not apply, VHHs refold easily and they are provided with increased solubility. Moreover, VHHs can easily be produced by bacteria or yeast allowing time and cost reduction during manufacturing.(35, 36) Furthermore, VHH domains are low immunogenic because of their high homology with human VH genes and the absence of the Fc-region.(29, 36) VHHs are ten times smaller than conventional antibodies, allowing them

to reach clefts in antigen structures and granting them with enhanced tissue penetration as compared with conventional antibodies.(37, 38)

Here, we describe the generation and evaluation of a bispecific VHH-based construct that combines inhibition of the epidermal growth factor receptor (EGFR)-signaling pathway via an antagonistic anti-EGFR VHH with the target-dependent activation of effector V γ 9V δ 2-T cells via an anti-V γ 9V δ 2-TCR VHH. V γ 9V δ 2-T cells activated in this manner produced pro-inflammatory cytokines such as IFN- γ and TNF- α and efficiently lysed EGFRexpressing tumor cell lines both *in vitro* and *in vivo*. This therapeutic effect was independent of *KRAS* or *BRAF* mutations, which are normally associated with resistance to anti-EGFR monoclonal antibody (mAb) therapy.(*39*, *40*) Moreover, variations in V γ 9V δ 2-TCR δ 2-CDR3 sequence that are known to be associated with reduced V γ 9V δ 2-T cell responses(*1*) to pAg stimulation stimulation did not affect cell killing efficacy. This novel bispecific VHHbased immunotherapeutic approach can be applied to many tumor types by simply replacing the tumor-specific VHH and does not require further individualization due to the conserved monomorphic nature of the V γ 9V δ 2-TCR.

Results

Selection of a human $V\gamma 9V\delta 2$ -TCR specific and -activating VHH

Vy9V82-TCR specific VHHs were generated by immunizing 2 lama glamas multiple times with human $V\gamma 9V\delta 2$ -T cells pooled from different healthy donors. Through phage display and after screening for V γ 9V δ 2-TCR specific fragments, 20 different V γ 9V δ 2-TCR specific VHHs were identified, either directed to the V δ 2- or to the V γ 9-chain, and either with activating or with non-activating potential as determined using a V γ 9V δ 2-TCR transduced JurMa luciferase reporter cell line.(32) The VHHs with activating potential identified in this screen were then tested for their capability to induce activation of human healthy donorderived V γ 9V δ 2-T cells via cross-linking. For this purpose, V γ 9V δ 2-T cells were cultured with plate-bound VHHs for 24 hrs. Activation of Vγ9Vδ2-T cells was determined by assessing up-regulation of the activation marker CD25, induction of CD107a expression, reflecting the release of cytotoxic granules, and the intracellular production of IFN-y as determined by flow cytometry. As a positive control we used NBP-pretreated HeLa cells. These screens led to the identification of the anti-V δ 2 VHH 6H4 and the anti-V γ 9 VHH 6H1 as the most consistently activating VHHs, inducing $V\gamma 9V\delta 2$ -T cell activation in all three assays across multiple donors (Fig. 1 A-C). Their ability to activate $V\gamma 9V\delta 2$ -T cells was further confirmed by studying the activation of Vy9V82-T cells from PBMC directly exvivo(data not shown).

Although the vast majority of $\gamma\delta$ -T cells in the human peripheral blood consist of V γ 9V δ 2-T cells, $\gamma\delta$ -T cells expressing either the V γ 9-chain (i.e. V γ 9⁺V δ 2⁻ $\gamma\delta$ -T cells) or the V δ 2-chain (i.e. V γ 9⁻V δ 2⁺ $\gamma\delta$ -T cells) exist; these, however, do not respond to pAg stimulation. As the relative frequency of V γ 9⁻V δ 2⁺ $\gamma\delta$ -T cells is very low and substantially lower than the level of V γ 9⁺V δ 2⁻ $\gamma\delta$ -T cells(*17*, *41*), we reasoned that a V δ 2-TCR chain specific VHH would more selectively target V γ 9V δ 2-T cells and would therefore be the preferred VHH to be used in a bispecific VHH construct aimed at the specific targeting of V γ 9V δ 2-T cells. For this reason, the V δ 2-TCR specific VHH 6H4 (Fig. 1D and 1E) was selected for further experiments.

To determine whether coupling of two anti-V γ 9V δ 2-TCR VHHs into one bivalent VHH construct could result in an even stronger activation of V γ 9V δ 2-T cells, two 6H4 VHH genes were engineered into one construct and separated by a flexible Gly₄Ser-linker (GS) of varying length (5-30 amino acids). First, bivalent VHH 6H4-5GS-6H4 was compared to the monovalent VHH 6H4 with respect to its ability to activate V γ 9V δ 2-T cells in a plate-bound

assay. At all tested concentrations, stimulation with the bivalent VHH resulted in a stronger V γ 9V δ 2-T cell activation as compared to the monovalent VHH (Fig. 1F). However, to be optimally effective in a tumor targeting construct, it is desirable that the VHH does not induce V γ 9V δ 2-T cell activation either on its own or in the absence of tumor cells. We observed that when V γ 9V δ 2-T cells were cultured with the bivalent VHH 6H4-5GS-6H4 added in solution, a strong V γ 9V δ 2-T cell activation was induced, even at very low concentrations (Fig. 1F). This was independent of the linker length between both 6H4 VHHs (data not shown). In contrast, when monovalent VHH 6H4 was added in solution to V γ 9V δ 2-T cells did not become activated (Fig. 1F). As V γ 9V δ 2-TCR cross-linking by the bivalent VHH constructs would likely result in non-specific systemic as opposed to target-specific activation of V γ 9V δ 2-T cells, the monovalent anti-V γ 9V δ 2 TCR VHH 6H4 was selected for incorporation into a bispecific tumor-targeting VHH construct.

Generation and functional evaluation of a bispecific anti-EGFR-anti-V γ 9V δ 2-TCR VHH construct

To generate a bispecific VHH construct, the anti-V γ 9V δ 2-TCR VHH 6H4 was joined to the previously generated and characterized high-affinity anti-EGFR VHH 7D12. This VHH is able to compete with EGF for EGFR binding and inhibits both EGFR phosphorylation and EGFR⁺ tumor cell proliferation in vitro and in vivo.(42, 43) To determine the optimal format with respect to binding and functionality, multiple bispecific VHH constructs were created, with variations in orientation and spacing between the individual VHHs. First, bispecific 7D12-6H4 and 6H4-7D12 VHH constructs were generated with a flexible Gly₄Ser-linker of 10 amino acids to determine whether target binding and affinity of the individual VHHs was maintained in the bispecific format and whether this was dependent on their orientation. Whereas the affinity of 6H4 to $V\gamma 9V\delta 2$ -T cells did not differ by its relative (i.e. N-terminal, or C-terminal) position in the bispecific VHH, the anti-EGFR 7D12 VHH clearly bound to EGFR⁺ A431 tumor cells more efficiently when it was positioned at the N-terminus (Fig. 2A). Therefore, the 7D12-6H4 format was considered optimal and variations of this bispecific VHH were made regarding linker length to assess whether this would influence binding efficiency and functionality. As shown in figure 2B, variations in linker length did not influence the binding of the construct to target cells. Furthermore, linker length differences did not influence the efficacy of V γ 9V δ 2-T cell activation, degranulation or tumor cell lysis induced by the bispecific VHH construct upon co-culture of $V\gamma 9V\delta 2$ -T cells

with EGFR-expressing tumor cells (Fig. 2C). This was also observed when the linker length was replaced to the smallest Gly_4 Ser-linker consisting of 5 amino acids (data not shown). As a small linker is least prone to proteolysis *in vivo*, we used the 5 amino acid (5GS) linker for all subsequent experiments.

V γ 9V δ 2-T cell activation and subsequent tumor cell lysis was formally demonstrated to depend on simultaneous binding of the bispecific VHH construct to V γ 9V δ 2-T cells and EGFR-expressing tumor cells by the use of control constructs incorporating the irrelevant VHH R2(44) (Fig. 3A). Of note, V γ 9V δ 2-T cell activation and degranulation levels induced by the 7D12-5GS-6H4 bispecific VHH construct were equivalent to those observed when V γ 9V δ 2-T cells were co-cultured with NBP-pretreated EGFR⁺ tumor cells. The maximum level of tumor cell lysis induced by V γ 9V δ 2-T cells was observed at concentrations as low as 10 nM of the 7D12-5GS-6H4 bispecific construct. Importantly, it was at least as effective as when tumor cells were pretreated with 100 μ M NBP. Furthermore, when titrating down the 7D12-5GS-6H4 concentration, efficient lysis of EGFR⁺ tumor cells was observed at concentrations as 10 pM in a 1:1 effector:target cell ratio. Importantly, this was not observed when the immortalized human B-cell line JY, lacking EGFR expression, was used as target (Fig. 3B), thus demonstrating the specificity of this targeting approach.

As treatment with anti-EGFR mAbs such as cetuximab or panitumumab is often accompanied by skin toxicity,(45, 46) we explored whether primary skin-derived (EGFR⁺) keratinocytes were lysed by $V\gamma9V\delta2$ -T cells in the presence of the 7D12-5GS-6H4 construct. EGFR expression was confirmed by flow cytometry demonstrating that the mean fluorescence index (MFI) of EGFR expression on keratinocytes was 3.2 ± 1.5 (*n*=3, mean \pm SD) (as a reference, the MFI of EGFR on A431 was 9.4, and the MFI of EGFR on JY was 0.8). Even at high concentrations of the bispecific targeting construct, only minor activation and cytolytic activity of $V\gamma9V\delta2$ -T cells was observed in the presence of primary keratinocytes (Supplementary fig. 1).

7D12-5GS-6H4 induces $V\gamma 9V\delta 2$ -T cell-mediated lysis of EGFR⁺ tumor cells irrespective of KRAS or BRAF mutation status

Of note, the 7D12 VHH retained its capacity to inhibit EGFR signaling in a dose-dependent manner upon incorporation into the bispecific VHH construct. As demonstrated by the analysis of EGFR phosphorylation in EGFR expressing tumor cells upon their exposure to EGF, this inhibitory activity was equivalent to that of the monovalent 7D12 VHH (Fig. 4A). To test if the 7D12-5GS-6H4 bispecific VHH exerted antitumor activity even in the presence

of activating mutations in the EGFR signaling pathway, $V\gamma 9V\delta 2$ -T cells were co-cultured with EGFR⁺ human colon cancer cell lines carrying either a mutation in *KRAS* (i.e. SW480 cells, expressing one of the most common and oncogenic *KRAS* mutations G12V) or *BRAF* (i.e. HT-29 cells, expressing the most common *BRAF* mutation V600E)(47, 48) in the presence or absence of 7D12-5GS-6H4. As shown in Fig. 4B-I, 7D12-5GS-6H4 induced potent V γ 9V δ 2-T cell activation, degranulation and tumor cell lysis of EGFR⁺ colon tumor cells, irrespective of their *KRAS* or *BRAF* mutation status.

7D12-5GS-6H4 activates $V\gamma 9V\delta 2$ -TCR-G115 with various $\delta 2$ -CDR3 sequence variations

The sequence and length of the δ 2-CDR3 region of the V γ 9V δ 2-TCR varies between individuals and in part determines the TCR affinity and cytolytic capacity upon binding of pAg expressing target cells.(1) Since VHH 6H4 specifically binds to the V δ 2-chain of the $V\gamma 9V\delta 2$ -TCR, we determined if common $V\delta 2$ -CDR3 variations influenced the binding of the VHH 6H4 to the V γ 9V δ 2-TCR and whether this affected its V γ 9V δ 2-T cell activating capacity. To this end, JurMa cells were transduced to express the wildtype $V\gamma 9V\delta 2$ -TCR-G115 or the V γ 9V δ 2-TCR-G115 with δ 2-CDR3 variations in the 98-103 region (Kabat numbering). This region was either replaced by i) a single alanine, creating a "short length" mutant ($\delta 2$ -G115_{LM1}) with complete abolishment of pAg/BTN3A1-reactivity; ii) 9 alanine amino acids, creating an "enlongated length" mutant (in $\delta 2$ -G115_{LM9}) with approximately 40% reduced pAg/BTN3A1-reactivity compared to wild-type Vγ9Vδ2-TCR-G115; iii) the δ 2-CDR3 sequence of the naturally weakly pAg/BTN3A1-reactive cl3 clone (δ 2-G115_{cl3}); or iiii) the δ 2-CDR3 sequence of the naturally highly pAg/BTN3A1-reactive cl5 clone (δ 2-G115_{c15}).(1) The δ 2-G115 length mutants and δ 2-G115_{c13} showed a slightly reduced binding of VHH 6H4 compared to $\delta 2$ -G115_{wT} and $\delta 2$ -G115_{c15} (Fig. 5A). However, and more importantly, no significant difference was observed in the ability of VHH 6H4 to trigger activation as determined by CD69 expression on the JurMa cells expressing the modified V γ 9V δ 2-TCRs as compared to the JurMa cells expressing the V γ 9V δ 2-TCR-G115_{wT} (Fig. 5B). Thus, donor sequence variations in δ 2-CDR3 that are known to impact pAg recognition, do not substantially affect the capacity of the 7D12-5GS-6H4 bispecific VHH construct to trigger V γ 9V δ 2-T cell activation and function.

7D12-5GS-6H4 enhances the $V\gamma 9V\delta 2$ -T cell-mediated inhibition of tumor outgrowth in vivo

We next assessed the effect of the 7D12-5GS-6H4 VHH on the outgrowth of human EGFRoverexpressing tumors in vivo. Immunodeficient BRGS^{wt} mice were engrafted with SW480^{Gluc} tumor cells carrying a KRAS^{G12V}-mutation and transduced to stably express Gaussia luciferase (Gluc). Expression of Gluc allowed real-time monitoring of viable primary and metastatic tumor cell load and response to treatment using bioluminescence imaging (BLI).(49) At days 1, 4, and 7, mice were treated with either PBS, cetuximab, Vy9V82-T cells, or Vγ9Vδ2-T cells in combination with 7D12-5GS-6H4 (Fig. 6A). At day 35, BLI clearly demonstrated that mice treated with the combination of Vy9V δ 2-T cells and 7D12-5GS-6H4 had an overall lower tumor burden compared to the mice treated with PBS, cetuximab or $V\gamma 9V\delta 2$ -T cells alone (Fig. 6B and C). Most importantly, and in accordance with the BLI data, mice treated with the combination of V γ 9V δ 2-T cells and 7D12-5GS-6H4 had a significantly improved overall survival compared with mice treated with either PBS, cetuximab or $V\gamma 9V\delta 2$ -T cells alone (Fig. 6D). These results confirm the expected treatment failure with cetuximab of KRAS mutated colorectal cancer cells and demonstrate that EGFRmediated tumor targeting by 7D12-5GS-6H4 enhances the Vy9V82-T cell-mediated inhibition of tumor outgrowth in vivo, even for cetuximab resistant (i.e. KRAS mutated) tumors.

Discussion

 $V\gamma 9V\delta 2$ -T cells have a unique combination of features that make them highly promising for use in cancer immunotherapy, i.e. the recognition of ligands exclusively exposed by stressed or altered cells in an MHC-independent manner, a rapid innate-like response, the ability to induce efficient target cell lysis via multiple routes (Fas/FasL and perforin pathway) against a wide variety of tumor targets, the induction of dendritic cell maturation, and even efficient antigen presentation to $\alpha\beta$ -T cells.(22, 25, 26) Several attempts have been made to clinically exploit $V\gamma 9V\delta 2$ -T cell activation in cancer patients but results thus far lack consistency.(27, 28) This is likely related to the absence of a specific trigger for the activated $V\gamma 9V\delta 2$ -T cells to home to and infiltrate tumor sites.

Here, we explored whether the antitumor activity of V γ 9V δ 2-T cells could be enhanced and directed to the tumor by using a bispecific VHH construct that would allow V γ 9V δ 2-T cell accumulation and activation specifically at the tumor site. As a model tumor antigen we selected EGFR, which is a key factor in epithelial malignancies as its activity enhances tumor growth, invasion, and metastasis.(50, 51) Agents aimed at EGFR inhibition, such as anti-EGFR mAbs competing for ligand binding (e.g. cetuximab and panitumumab) and EGFR-specific tyrosine kinase inhibitors (TKI; e.g. erlotinib or gefitinib), are currently registered treatments for various advanced-stage epithelial cancers, including non-small-cell lung cancer, colorectal cancer, pancreatic cancer, and head and neck squamous cell carcinoma.(52) Treatment with these agents is related to improved progression free and overall survival, though the overall efficacy is generally limited and frequently restricted to certain patient subsets, leaving ample room for improvement.(52)

From a set of 20 V γ 9V δ 2-TCR specific VHHs generated from immunized llamas and selected by phage display, we selected the V δ 2-specific VHH 6H4 on the grounds that it consistently induced V γ 9V δ 2-T cell activation and since targeting the V δ 2-chain would be more specific for V γ 9V δ 2-T cells than targeting the V γ 9-chain, as in general V γ 9⁺V δ 2⁻ $\gamma\delta$ -T cells are more abundant than V γ 9·V δ 2⁺ $\gamma\delta$ -T cells in the human peripheral blood.(*17*, *41*) As bivalent formats of the V δ 2-specific VHH 6H4 already induced striking activation of V γ 9V δ 2-T cells in the absence of target cells, probably due to the crosslinking of the TCRs, and this was expected to result in systemic activation of V γ 9V δ 2-T cells when applied therapeutically, we decided to use the monovalent VHH 6H4 for incorporation in the bispecific VHH in order to minimize the chances of non-specific (tumor unrelated) activation upon systemic administration. By joining an anti-EGFR VHH (7D12) to the 6H4 VHH, we created a bispecific VHH construct targeting both EGFR and the $V\gamma 9V\delta 2$ -TCR. The generated 7D12-5GS-6H4 bispecific VHH induced strong activation and degranulation of Vy9V82-T cells resulting in potent lysis of EGFR expressing tumors at picomolar concentrations in an EGFR and $V\gamma 9V\delta 2$ -TCR dependent fashion. Previously, we determined that VHH 7D12 inhibited EGFR phosphorylation and pathway activation by binding to EGFR domain III, thus preventing its conformational change to an active state.(42, 43) Importantly, integration of anti-EGFR VHH 7D12 into the bispecific format did not alter its ability to inhibit EGF-induced signaling in EGFR overexpressing cancer cells. Moreover, and in contrast to the currently available anti-EGFR mAb therapies which are mainly effective through the inhibition of EGFR signaling(52), the 7D12-5GS-6H4 bispecific VHH construct described here also induced efficient Vy9V82-T cell-mediated lysis of colorectal cancer cell lines carrying common KRAS or BRAF mutations. Mutations such as these in the protooncogenes of the RAS family (e.g. KRAS, NRAS, HRAS and BRAF) frequently occur in e.g. colorectal, pancreatic and lung cancers, which together account for a major proportion of cancer cases. This often makes tumors resistant to the currently available anti-EGFR therapies (e.g. mAbs and TKI)(39, 40) and leads to poor prognosis(53, 54). Our data demonstrate that 7D12-5GS-6H4 has a dual mechanism of action by combining the inhibition of EGF-induced signaling (involved in tumor survival, growth and metastasis) with the direct induction of tumor cell lysis. The effective and superior anti-tumor effect of the bispecific VHH construct was confirmed using mice xenograft experiments. Mice grafted with human EGFR-overexpressing KRAS-mutated tumor cells that were treated with the bispecific VHH in combination with V γ 9V δ 2-T cells showed significant reduction of tumor outgrowth and improved overall survival. These are promising results that suggest that the bispecific 7D12-5GS-6H4 VHH construct may also inhibit growth of EGFR⁺ tumors in patients independent of the RAS or BRAF mutation status of the tumor and might thereby allow a more widespread applicability of EGFR-targeted treatments by bypassing the need for RAS and BRAF mutation analyses.(55) Moreover, the data presented here provide a proof of concept for $V\gamma 9V\delta 2$ -T cell targeted therapy for a broad range of tumor types, which may be facilitated by simply exchanging the anti-EGFR VHH for VHHs targeting various other tumor antigens.

Interestingly, the ability to overcome the therapeutic barrier posed by *RAS* mutations was also noted for bispecific T cell engagers (BiTEs) wherein the scFv variable domains of cetuximab or panitumumab were fused to a scFv against CD3 expressed by T cells.(56) However, though some BiTEs have induced clinical responses, limitations of this approach

include the requirement for continuous infusions of the drug due to its short serum half-life time and the fact that it targets CD3 which is expressed by all T cells including immunosuppressive T cells such as Tregs.(29) Tregs predominate in the tumor microenvironment, actively suppress the activation and proliferation of effector T cells and are related to unfavorable prognosis.(30) For this reason, antibody-based constructs designed to exclusively trigger immune cells with a pro-inflammatory function, such as V γ 9V δ 2-T cells, may be preferable over the targeting of CD3.(31)

In a recently published preclinical study the antitumor efficacy of $V\gamma 9V\delta 2$ -T cells was explored through the use of a tribody targeting the γ 9-chain of the V γ 9V δ 2-TCR and the antigen Her2/neu. This (Her2)2xVy9 tribody efficiently lysed Her2/neu tumor overexpressing pancreatic cells in vitro and in mouse xenografts.(57) Although this nearly full-sized (~100kD) antibody approach underscores the potential of tumor-targeted V γ 9V δ 2-T cell-based immunotherapies, more specific targeting of the V γ 9V δ 2-T cell population can be achieved using an antibody (fragment) directed to V δ 2 compared to V γ 9, as in our bispecific VHH. Furthermore, the development and use of whole mouse mAbs has several limitations including the mispairing of heavy and light chains and the risk of developing human-anti-mouse antibodies (HAMA) in patients which leads to antibody neutralization and adverse events in the form of a cytokine release syndrome.(29, 36) These limitations can be overcome by the use of VHHs. VHH are low immunogenic because they share high homology with human VH genes and are devoid of an Fc-region. (29, 33, 36) Because of the single domain nature of VHHs, pairing issues do not apply. This advantage in combination with their small size and the fact that they do not require post-translational modifications, allow VHHs to be easily produced in bacteria or yeast, which remain the most cost- and timeefficient production systems to date.(36, 58, 59) VHHs are known for their high stability at elevated temperature and pH, providing them with enhanced solubility and making them less prone to aggregation.(36, 60) The small size of VHHs (~30 kDa for a bispecific VHH) also facilitates deep tumor tissue penetration compared to larger sized antibody constructs, but like other small antibody fragments (such as BiTEs) this is also associated with a short serum half-life time due to fast renal clearance. This can be circumvented by fusion of the VHH to a serum albumin binding VHH.(29, 38, 42, 61)

As anti-EGFR mAb therapy can be complicated by (generally well manageable) skin toxicity as a result of mAb binding to EGFR expressed on keratinocytes, it was encouraging to see only minor keratinocyte lysis when keratinocytes were cultured with $V\gamma 9V\delta 2$ -T cells

in the presence of the 7D12-5GS-6H4 bispecific VHH. Although preclinical tests such as this do not necessarily predict safety in patients, these results are encouraging with regards to the future clinical exploration of this particular bispecific VHH construct.

In conclusion, we here describe the development of a bispecific VHH construct with a dual mechanism of action, combining ligand deprivation crucial for tumor cell proliferation and survival with the efficient and exclusive lysis of EGFR expressing tumor cells by conserved immune effector $V\gamma 9V\delta 2$ -T cells. Since EGFR is a widely expressed and clinically validated tumor antigen, a large patient group could benefit from this therapy. This group can be even broadened by the fact that, in contrast to currently available anti-EGFR therapies, the effectiveness of this therapy will not be influenced by downstream mutations in e.g. *RAS* or *BRAF* and, due to the monomorphic nature of the $V\gamma 9V\delta 2$ -TCR this immunotherapeutic approach requires no further individualization. Furthermore, as recently VHHs directed to various other tumor antigens have been developed(*62*) and continue to be developed, these can easily be exchanged for the anti-EGFR VHH enabling future $V\gamma 9V\delta 2$ -T cell targeted therapy for a broad range of tumor types.
Materials & methods

Cell lines

HeLa, A431, HT29, Colo320 and SW480 cell lines were obtained from ATCC and, as well as Her14(*63*) cultured in DMEM+, i.e. Dulbecco's Modified Eagle's Medium (Lonza, catalog #BE12-614F) supplemented with 10% (v/v) fetal calf serum (FCS) (HyClone GE Healthcare, catalog #SV30160.03), 100 IU/mL sodium penicillin, 100 µg/mL streptomycin sulfate and 2.0 mM L-glutamine (Life Technologies, catalog #10378-016).

The SW480 cell line was stably transduced with lentivirus carrying the Gaussia Luciferase (Gluc) and Cerulean Fluorescent Protein (CFP) genes (LV-CFP-Gluc)(64), kindly provided by Tom Würdinger (VU University medical center (VUmc), Amsterdam, NL), to generate the SW480^{Gluc} cell line. CFP positive SW480^{Gluc} cells were sorted and used for tumor injection in mice when CFP expression was >95% as determined by flow cytometry.

JY cells and Jurkat transductants(32) were cultured in IMDM+, i.e. Iscove's modified Dulbecco's medium (Lonza, catalog #BE12-722F) supplemented with 10% (v/v) FCS, 0.05 mM β -mercaptoethanol, 100 IU/mL sodium penicillin, 100 µg/mL streptomycin sulfate and 2.0 mM L-glutamine. JurMa cell lines transduced with V γ 9V δ 2-TCR-G115_{WT} and δ 2-CDR3 variants, were generated as previously described(1) and cultured in IMDM+.

Keratinocytes were isolated from human adult skin as described previously.(65) In brief, epidermal sheets were separated from dermis by incubation with Dispase II (Roche, catalog #04942078001) overnight at 4°C. Subsequently, keratinocytes were isolated from the epidermis by a 10 minute 0.125% trypsin incubation (HyClone GE Healthcare, catalog # SH3004201) and seeded per 3*10⁶ cells in keratinocyte culture medium on 9-cm-diameter tissue culture dishes coated with 0.5 µg/cm² human placental collagen IV (Sigma-Aldrich, catalog #C5533). Keratinocyte culture medium consisted of Dulbecco's Modified Eagle's Medium and Ham's F12 (Invitrogen, catalog #21765-029) in a 3:1 ratio, supplemented with 10% (v/v) FCS, 100 IU/mL sodium penicillin, 100 µg/mL streptomycin sulfate, 2.0 mM L-glutamine, 1 µmol/L hydrocortisone (Sigma-Aldrich, catalog #H0888), 1 µmol/L isoproterenol hydrochloride (Sigma-Aldrich, catalog #I6504), 0.09 µmol/L insulin (Sigma-Aldrich, catalog #15500), and 2 ng/ml human keratinocyte growth factor (Sigma-Aldrich, catalog #K1757). All cell lines were maintained at 37°C with 5% CO₂ in a humidified atmosphere and tested mycoplasma negative.

Flow cytometry and monoclonal antibodies

FITC-labeled anti-TCR Vδ2 (catalog #555738), FITC-labeled anti-IFN-γ (catalog #554700), FITC-labeled anti-CD69 (catalog #347823), PE-labeled anti-CD107a (catalog #555801), PElabeled anti-CD25 (catalog #55542), PE-labeled pan γδ-TCR (catalog #333141), APClabeled anti-CD25 (catalog #340907), and 7-AAD (catalog #559925) were obtained from BD Biosciences. PerCP-labeled anti-TCR Vδ2 (catalog #331410), PE-labeled anti-TCR Vγ9 (catalog #331308) and APC-labeled anti-TCR Vγ9 (catalog #331310) were from Biolegend. RPE-labeled goat-anti-mouse F(ab')2 fragment (catalog #R0480) was obtained from Dako. Anti-Myc tag mAb clone 4A6 (catalog #05-724) was obtained from Merck Millipore and anti-Myc tag mAb clone 9E10 was produced in-house. Alexa488-labeled cetuximab was a kind gift of Rens Braster and Yvette van Kooyk (VUmc, Amsterdam, NL). All stainings for flow cytometry were performed in PBS supplemented with 0.1% BSA and 0.02% sodiumazide. Intracellular IFN- γ production was determined by adding GolgiPlug to the cell culture for the final 4 hrs of the experiment. Cells were fixed and permeablized with the Fixation/Permeabilization Solution Kit from BD Biosciences(catalog #555028) and stained with anti-IFN- γ mAb.

Stained cells were measured with a FACS Calibur or LSRFortessa (BD Biosciences) and analyzed with CellQuest (BD Biosciences) or Kaluza software (Beckman-Coulter).

Generation of donor-derived V γ 9V δ 2-T and pan $\gamma\delta$ -T cell lines

Healthy donor-derived V γ 9V δ 2-T cells were isolated, expanded and cultured as described previously.(*32*) In brief, V γ 9V δ 2-T cells were isolated by magnetic-activated cell sorting from PBMC using FITC-labeled anti-TCR V δ 2 or PE-labeled anti-TCR V γ 9 mAb in combination with anti-mouse IgG MicroBeads (Miltenyi Biotec, catalog #130-048-401). Purified V γ 9V δ 2-T cells were stimulated weekly with irradiated and NBP-pretreated (100 μ M Pamidronate for 2-3 hrs, Teva Pharmachemie, catalog #12J08RD) human mature monocyte derived dendritic cells or an irradiated feeder mix (PBMC of 2 healthy human donors and Epstein Barr Virus transformed B cells with addition of 50 ng/ml PHA). V γ 9V δ 2-T cells were used for experiments when V γ 9⁺V δ 2⁺-TCR expression was >90% and CD25 expression was <40% as determined by flow cytometry.

 $V\gamma9V\delta2$ -T cell lines were cultured in Yssels+, i.e. Yssels medium(66) supplemented with 1% human AB serum (Cellect, MP Biomedicals, catalog #2931949), 50 U/ml rhIL-2 (Proleukin, Novartis), 0.05 mM β -ME, 100 IU/mL sodium penicillin, 100 μ g/mL

streptomycin sulfate and 2.0 mM L-glutamine. V γ 9V δ 2-T cell lines and tumor target cell lines were cultured in IMDM+ medium during experiments. The V γ 9V δ 2-T cell lines were maintained at 37°C with 5% CO₂ in a humidified atmosphere and tested mycoplasma negative.

 $V\gamma 9^+V\delta 2^+$, $V\gamma 9^+V\delta 2^-$, $V\gamma 9^+V\delta 2^+$ and $V\gamma 9^-V\delta 2^-\gamma \delta$ -T cell lines were obtained from human PBMC by MACS isolation. PBMC were stained with PE-labeled pan $\gamma\delta$ -TCR antibody and anti-mouse IgG MicroBeads. This pan $\gamma\delta$ -T cell line was expanded with a feeder mix and subsequently stained with FITC-labeled anti-TCR V δ 2 and PE-labeled anti-TCR V γ 9 antibodies to allow flow cytometric cell sorting of 4 separate populations (i.e. $V\gamma 9^-V\delta 2^+, V\gamma 9^+V\delta 2^-, V\gamma 9^+V\delta 2^+$ and $V\gamma 9^-V\delta 2^-\gamma \delta$ -T cells).

Generation, production and purification of bivalent and bispecific VHHs

To generate bivalent or bispecific VHHs, genes of VHH 6H4(*32*), VHH 7D12(*42*) or VHH R2(*44*) were PCR-amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, catalog #M0530) and appropriate primers encoding the N- or C-terminal end of the VHH gene, a restriction endonuclease site, and a linker sequence (composed of Gly4-Ser repeats). PCR products were purified by gel extraction using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, catalog #740609), digested with restriction endonucleases to allow cloning into appropriate plasmids.

For *in vitro* experiments, PCR products were cloned to plasmid pMek219, verified by sequencing and produced in TG1 bacteria as described previously(*32*). Produced VHH were derived from the bacterial periplasm by a PBS freeze-thawing step and purified by immobilized metal ion affinity chromatography (IMAC) using Talon resin (Clontech, catalog # 635504). VHH were eluted with 150 mM imidazole and dialyzed twice against PBS.

For *in vivo* experiments, PCR products were cloned to a modified version of the pFastBac I plasmid (Thermo Fisher Scientific) containing a Honeybee Melittin Signal Sequence (HMSS) and a C-terminal his-tag. Bacmid DNA and virus were essentially prepared according to the Bac-to-Bac manual (Thermo Fisher Scientific). Briefly, the pFastBac constructs were transformed into EMBACY cells(*67*) and bacmid DNA was isolated. Sf9 insect cells were transfected with the bacmid DNA and the virus was subsequently amplified in a sf9 suspension culture. Collected virus was used to infect sf9 cells for protein expression. Medium containing the secreted proteins was harvested 3 days post infection and dialyzed 2 x against 25 mM HEPES pH 7.5 and 200 mM NaCl. Proteins

were purified on a 5 ml HiTrap Ni2+ column, eluted with 200 mM imidazol in 25 mM HEPES pH 7.5, 200 mM NaCl. Proteins were further purified by size exclusion chromatography on a S75 16/60 Superdex column (GE Healthcare) equilibrated with PBS buffer. Fractions containing the proteins were pooled, concentrated and passed through a 0.22 μ M filter.

The purity of produced protein was checked on a coomassie blue-stained protein gel before use.

Binding analysis of VHH

To determine the binding of VHH to cells, $5*10^4$ V γ 9V δ 2-T cells, A431 cells, Jurkat or JurMa transductants were incubated with VHH at the indicated concentrations for 30 minutes. Bound VHH was detected with anti-Myc-tag antibody clone 4A6 and RPE-labeled goat-anti-mouse F(ab')2 fragment by flow cytometry.

To determine the binding persistence and stability of bivalent VHH 7D12-5GS-6H4 to V γ 9V δ 2-T cells, V γ 9V δ 2-T cells were incubated with 100 nM VHH for 30 minutes, unbound VHH was washed away and the cells were cultured for 0, 8 or 15 days in Yssels+ in the presence of 10 U/ml rhIL-2 (Proleukin, Novartis). On days 0, 8 and 15 a sample was taken from the culture and VHH bound to the V γ 9V δ 2-T cells was detected with anti-Myc-tag antibody clone 4A6 and an RPE-labeled goat-anti-mouse F(ab')2 fragment by flow cytometry.

Functional analysis of monospecific, bivalent and bispecific VHH

To determine the effect of monovalent and bivalent V γ 9V δ 2-TCR specific VHH on V γ 9V δ 2-T cell activation, V γ 9V δ 2-TCR expressing cells were either cultured in the presence of plate bound or soluble VHHs. For plate bound conditions, wells of a 96-well flatbottom culture plate (Greiner, catalog #655180) were coated with 4 μ g/ml mouse-anti-Myc clone 9E10 in PBS overnight at 4°C. Wells were washed three times with PBS and incubated for 2 hrs with the indicated concentrations of VHH in PBS. After the wells were again washed three times with PBS, 10⁵ V γ 9V δ 2-T cells, 10⁵ V γ 9V δ 2-TCR-G115 transduced JurMa cells or 2.5*10⁵ PBMC were added per well in a final volume of 200 μ 1 IMDM+. For soluble VHH conditions, the indicated concentration of VHH in PBS was added to 10⁵ V γ 9V δ 2-T cells in a 96-well flat-bottom culture plate. For control conditions, V γ 9V δ 2-T cells were co-cultured with untreated (negative control) or NBP-treated (100 μ M Pamidronate for 2-3 hrs, positive control) HeLa cells in a 1:1 ratio. Cells were cultured in a final volume of 200 μ 1 IMDM+ for 24 hrs.

To determine the effect of bispecific anti-EGFR-anti-V γ 9V δ 2-TCR VHH on V γ 9V δ 2-T cell activation, degranulation and target cell lysis in a 24-hour assay, 5*10⁴ target cells (A431, JY, HT29, Colo320 or SW480) were labeled with 40 nM CFSE (Sigma-Aldrich, catalog #21888) or 5 μ M PBSE (Thermo Fisher Scientific, catalog #P10163), according to the manufacturer`s protocol, and allowed to adhere for 4 hrs in a 96-well flat bottom culture well. V γ 9V δ 2-T cells were incubated with the indicated concentrations of VHH for 1 hr at 4°C, washed three times with PBS and added in a 1:1 ratio to the target cells and cultured for 24 hrs in a final volume of 200 μ 1 IMDM+. In case of keratinocytes, primary keratinocytes were plated 2 days beforehand on collagen IV coated wells to obtain a viable cell pool at the start of the experiment. For the NBP-pretreated positive control, target cells were incubated with 100 μ M Pamidronate during cell adherence and washed by a 3x PBS rinse before the addition of V γ 9V δ 2-T cells.

To determine the biological efficacy of the 7D12-5GS-6H4 bispecific VHH or control monovalent R2 VHH over time, V γ 9V δ 2-T cells were incubated with 100 nM VHH for 30 minutes, unbound VHH was washed away and the cells were cultured for 15 days in Yssels+. A final concentration of 10 U/ml rhIL-2 was added to the culture every 3 days. On day 15, V γ 9V δ 2-T cells were transferred in a 1:1 ratio to a 96-well flat bottom culture well containing 4 hr adhered A431 cells. Cells were co-cultured for 24 hrs in a final volume of 200 μ 1 IMDM+.

To determine degranulation of V γ 9V δ 2-T cells, anti-CD107a mAb and GolgiStop (BD Bioscience, catalog #554724) were added to the co-culture for the final 4 hrs of the experiment. At the end of the experiment, cells were harvested and stained with anti-V δ 2 and/or anti-V γ 9 mAb or CD3 mAb to identify V γ 9⁺V δ 2⁺-T cells. CD25 and CD69 expression on V γ 9V δ 2-T cells was determined with an anti-CD25 and anti-CD69 mAb, respectively. Cells were stained with 7-AAD according to the manufacturer`s protocol to distinguish lysed cells. mAbs bound to the cells and 7-AAD staining were analyzed by flow cytometry.

Inhibition of EGF-induced EGFR phosphorylation

Inhibition of EGF-induced EGFR phosphorylation was performed as described before.(68) 10⁵ Her14 cells were seeded per well in a 12-wells plate in DMEM+ and allowed to adhere. DMEM^{min}. After 8 hrs. the medium was replaced by i.e. Dulbecco's Modified Eagle's Medium supplemented with 0.1% (v/v) FCS, 100 IU/mL sodium penicillin, 100 µg/mL streptomycin sulfate and 2.0 mM L-glutamine. The following day, cells were washed once with PBS, after which a mixture of the indicated VHHs was added to the cells in combination with 8 nM recombinant human EGF (Peprotech, catalog # AF-100-15) in DMEM^{min} for 15 minutes at 37°C. Subsequently the cells were washed three times with icecold PBS and resuspended in 2x Laemmli protein sample buffer. Half of the sample was loaded and run on a SDS-PAGE gel and western blotted. Phosphorylated EGFR was detected with an anti-phosphoEGF Receptor (Tyr1068) polyclonal antibody and an anti-rabbit-HRP mAb (both from Cell Signaling Technology, catalog #2234 and #7074, respectively). Blots were stained with an anti-β-actin (Sigma-Aldrich, catalog #21888) and anti-mouse HRP mAb (Cell Signaling Technology, catalog #7076) to demonstrate that equal amounts of cell lysate were loaded on gel.

In vivo studies

Immunodeficient BRGS mice (BALB/c $Rag2^{-/}ll2rg^{-/}Sirpa^{NOD}$)(69) were housed in isolators under pathogen-free conditions and randomly divided in 4 treatment groups (n=6/group). At day 0, mice received an intravenous (i.v.) tail vein injection with 0.5*10⁶ SW480^{Gluc} cells. At days 1, 4 and 7 mice were treated with either a) 500 µg cetuximab i.p., b) 1*10⁷ V γ 9V δ 2-T cells i.v., c) 1*10⁷ V γ 9V δ 2-T cells in combination with 1 µg of the bispecific 7D12-5GS-6H4 VHH i.v., or an equal volume of sterile PBS i.v. Mice that received V γ 9V δ 2-T cells were injected at days 1, 4, 7, 10 and 14 with 10,000 U human recombinant IL-2 i.p. to stimulate the proliferation of activated V γ 9V δ 2-T cells. Bioluminescence imaging (BLI) was performed at day 35 in 4 randomly selected mice from each study group. For this procedure, mice were anesthetized with inhalation anesthetics (isofluorane/oxygen) and injected i.v. retro-orbitally with 4 mg/kg coelenterazine (PJK GmbH, native-CTZ). BLI was recorded using an IVIS imaging system (PerkinElmer) and images were analysed using Living Image 4.0 software. BRGS mouse experiments were approved by the animal ethical committee of the Institut Pasteur (Reference # 2007–006), Paris, France, and validated by the French Ministry of Education and Research (Reference # 02162.01).

Statistical analyses

Statistical analyses were performed in GraphPad Prism version 5 (La Jolla, CA, USA). For *in vitro* analyses, a one-way ANOVA with a Bonferroni's post-hoc test was used. For *in vivo* data analysis of BLI, an unpaired T-test was used. For the survival analysis, a Mantel-Cox test was used. All findings were considered significant when *p*-values were <0.05.

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Authorship Contributions

RB performed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript.

SL performed research, analyzed and interpreted data and performed statistical analysis and wrote the manuscript.

FS performed research, analyzed and interpreted data and wrote the manuscript SLL performed research, analyzed and interpreted data and wrote the manuscript JV performed research, analyzed and interpreted data and wrote the manuscript RL performed research, analyzed and interpreted data and wrote the manuscript AS performed research, analyzed and interpreted data and wrote the manuscript ZS designed research, performed research and revised the manuscript critically JK designed research, interpreted data and revised the manuscript critically CM designed research and revised the manuscript critically EH designed research and revised the manuscript critically RR designed research and revised the manuscript critically JD designed research and revised the manuscript critically PBH designed research and revised the manuscript critically HMWV designed research and revised the manuscript critically TD designed research, analyzed and interpreted data, and wrote the manuscript HJV designed research, analyzed and interpreted data, and wrote the manuscript

Conflict of interest

We would like to state that authors Renée de Bruin, Tanja de Gruijl, Henk Verheul and Hans van der Vliet have a patent on V γ 9V δ 2-TCR specific nanobodies. Author Jürgen Kuball reports grants from Pierre Fabre, Novartis, and Miltenyi Biotech. He is scientific co-founder and CSO of gadeta (www.gadeta.nl) and inventor of multiple patents on $\gamma\delta$ TCR receptors and isolation strategies for engineered immune cells. The other authors have no financial conflicts of interest.

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Figures



Figure 1. Characteristics of Vγ9Vδ2-T cell activating VHH. A-C) Vγ9Vδ2-T cells were cultured with individual plate bound (wells coated with 500 nM) anti-Vγ9Vδ2-TCR VHH, control VHH R2, HeLa cells or NBP-pretreated HeLa cells in a 1:1 ratio. After 24 hrs, Vγ9Vδ2-T cell activation was determined by assessing the percentage of Vγ9Vδ2-T cells positive for A) CD25, B) CD107a, or C) intracellular IFN-γ using flow cytometry. Shown are means subtracted by background levels ± SEM of *n*=3-5 experiments. *p*-Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test. (* indicates *p*<0.05 and ** indicates *p*<0.01). D) The anti-Vδ2 VHH 6H4 (40 nM) binds to Jurkat-Vγ9Vδ2-TCR cells (*thick line*), but not to Jurkat cells without TCR expression (*filled grey*) or Jurkat-Vα24Vβ11-TCR cells (*dotted line*). E) The anti-Vδ2 VHH 6H4 (350 nM) binds to healthy donor-derived Vγ9⁺Vδ2⁺ (*thick line*) and Vγ9⁻Vδ2⁺ γδ-T cells but not to Vγ9⁻Vδ2⁻ (*filled grey*) or Vγ9⁺Vδ2⁻ γδ-T cells (*dotted line*). F) Vγ9Vδ2-T cells were cultured with plate

bound or soluble monovalent VHH (*filled squares*), bivalent VHH (*filled triangles*) or control VHH R2 (*open circles*) at the indicated concentrations for 24 hrs. Expression of CD25 was assessed using flow cytometry. Representative figures of n=3 experiments are shown. Abbreviations: aminobisphosphonates (NBP); Gly₄Ser (GS).





Figure 2. The effect of orientation and linker length in the bispecific VHH. A and B) $V\gamma9V\delta2$ -T cells (*left*) or EGFR-expressing A431 cells (*right*) were incubated in the presence or absence of the indicated VHHs and bound VHH was assessed by flow cytometry. Mean fluorescence intensity (MF) of bound VHH to the cells is depicted. C) $V\gamma9V\delta2$ -T cells and A431 cells were co-cultured in a 1:1 ratio for 24 hrs in the presence or absence of the indicated bispecific VHH. Both CD25 (*left*) and CD107a (*middle*) expression on $V\gamma9V\delta2$ -T cells were assessed by flow cytometry. The percentage of lysed A431 cells (*right*) was determined using 7-AAD staining and flow cytometry. Representative figures of n=3 experiments are shown. Abbreviations: Gly₄Ser (GS).

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Figure 3. The 7D12-5GS-6H4 bispecific VHH induces Vγ9Vδ2-T cell activation and lysis of EGFR expressing tumor cells. $V\gamma 9V\delta 2$ -T cells were cultured with or without EGFR⁺A431 tumor cells (A-B) or EGFR⁻JY cells (B) in a 1:1 ratio in the presence of the 7D12-5GS-6H4 bispecific VHH or a bispecific control VHH. VHH concentrations: A) 10 nM; B) as indicated. For control situations, $V\gamma 9V\delta 2$ -T cells were co-cultured with target cells in the absence of VHH (no VHH; negative control) or with NBP-pretreated target cells (positive control). After 24 hrs, $V\gamma 9V\delta 2$ -T cell activation and degranulation was determined by assessing the percentage of CD25 or CD107a expression, respectively by flow cytometry. The percentage of lysed target cells was determined using 7-AAD staining and flow cytometry. A) White bars represent $V\gamma 9V\delta 2$ -T cell mono-cultures in the absence of VHH, grey bars represent target cell mono-cultures in the absence of VHH and black bars represent Vy9V82-T cell co-cultures with target cells and indicated VHH. B) Co-cultures of target cells with $V\gamma 9V\delta 2$ -T cells and indicated amount of 7D12-5GS-6H4 bispecific VHH. Shown are mean \pm SEM of *n*=3-4 experiments. *p*-Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (* indicates p<0.05 and *** indicates p<0.001). Abbreviations: aminobisphosphonates (NBP); Gly₄Ser (GS).

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Figure 4



Figure 4. The 7D12-5GS-6H4 bispecific VHH inhibits EGFR signaling but does not depend on this to induce tumor cell lysis. A) The anti-EGFR 7D12 VHH retains its capacity to inhibit phosphorylation of EGFR when incorporated in a bispecific 7D12-5GS-6H4 VHH format. Her14 cells were incubated with a mixture of 8 nM human EGF and the indicated VHH. Cell lysates were run on SDS-PAGE gel and western blotted for phosphorylated EGFR_{Tyr1068} and β -actin as a loading control. B-I) V γ 9V δ 2-T cells were cultured with or without the EGFR⁺ colon tumor cells SW480 *KRAS^{G12V}* (B-E) or HT29 *BRAF^{V600E}* (F-I) in a 1:1 ratio in the presence of the 7D12-5GS-6H4 bispecific VHH or a bispecific control VHH. VHH concentrations: B-D and F-H) 10 nM; E and I) as indicated. For control situations, V γ 9V δ 2-T cells were co-cultured with target cells in the absence of VHH (no VHH; negative control) or with NBP-pretreated target cells (positive control). After 24 hrs, V γ 9V δ 2-T cell activation and degranulation was determined by assessing the percentage of CD25 (B and F) or CD107a (C and G) expression, respectively by flow

cytometry. The percentage of lysed target cells was determined using 7-AAD staining and flow cytometry (D-E and H-I). B-D and F-H) *White bars* represent V γ 9V δ 2-T cell monocultures in the absence of VHH, grey bars represent target cell mono-cultures in the absence of VHH and *black bars* represent V γ 9V δ 2-T cell co-cultures with target cells and indicated VHH. E and I) Co-cultures of target cells with V γ 9V δ 2-T cells and the indicated amount of 7D12-5GS-6H4 bispecific VHH. Shown are means ± SEM of *n*=3 experiments. *p*-Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (* indicates *p*<0.05, ** indicates *p*<0.01 and *** indicates *p*<0.001). Abbreviations: aminobisphosphonates (NBP); Gly₄Ser (GS).



Figure 5. The anti-V γ 9V δ 2-TCR specific VHH 6H4 efficiently activates V γ 9V δ 2-T cells harbouring δ 2-CDR variations. A) Indicated JurMa transductants were incubated with 500 nM VHH 6H4 and bound VHH was assessed by flow cytometry. Mean fluorescence intensity (MF) of VHH bound to the cells is depicted. A representative figure of *n*=3 experiments is shown. B) Indicated JurMa transductants were cultured with HeLa cells (negative control, *white*), NBP-pretreated HeLa cells (positive control, *grey*) or plate bound (wells coated with 500 nM) VHH 6H4 (*black*). After 24 hrs, the activation status of the cells was determined by assessing CD69 expression on the cells by flow cytometry. Indicated significant differences are relative to values of δ 2-G115_{WT} cells stimulated with HeLa cells. A representative figure of triplicate samples (mean ± SEM) of *n*=3 experiments is shown. *p*-Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (*** indicates *p*<0.001). Abbreviations: aminobisphosphonates (NBP).

Figure 6



Figure 6. The 7D12-5GS-6H4 bispecific VHH inhibits tumor growth *in vivo*. Immunodeficient BRGS mice grafted with SW480^{*Gluc*} cells were treated with PBS (control group; A), cetuximab (500 μ g i.p.; group B), V γ 9V δ 2-T cells (1*10⁷ i.v.; group C) or V γ 9V δ 2-T cells and 7D12-5GS-6H4 VHH (1*10⁷ and 1 μ g, respectively, both i.v.; group D) at days 1, 4 and 7. IL-2 (10,000 U, i.p.) was administered on days 1, 4, 7, 10 and 14 to the groups receiving V γ 9V δ 2-T cells. A) A schematic overview of the treatment schedule. B and C) Bioluminescence imaging at day 35 of 4 mice per treatment group. B) Heat map indicating the sites and relative level of tumor cell activity in individual mice. Red squares indicate the image field used for quantification analysis. C) Quantified bioluminescence signal measured per mouse expressed as the measured radiance normalized to the number of pixels, time and angle of imaging. Shown are means ± SEM of n=4 mice per group. *p*-Values

were calculated with a unpaired T-test (* indicates p<0.05). D) Kaplan-Meier analyses of mouse survival, n=6 mice per group. p-Values were calculated with a Mantel-Cox test (* indicates p<0.05, ** indicates p<0.01 and *** indicates p<0.001). Abbreviations: Gly₄Ser (GS).

Supplementary figures

Supplementary figure 1



Supplementary figure 1. The 7D12-5GS-6H4 bispecific VHH induces minimal lysis of keratinocytes. V γ 9V δ 2-T cells were cultured with or without primary keratinocytes in a 1:1 ratio in the presence of the 7D12-5GS-6H4 bispecific VHH or a bispecific control VHH. VHH concentrations: A) 10 nM and B) as indicated. After 24 hrs, the percentage of lysed keratinocytes cells was determined using 7-AAD staining and flow cytometry. A-B) *White bars* represent V γ 9V δ 2-T cell mono-cultures in the absence of VHH and *black bars* represent V γ 9V δ 2-T cell co-cultures with primary keratinocytes and indicated VHH. Shown are means \pm SEM of *n*=3 experiments. *p*-Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (* indicates *p*<0.05). Abbreviations: aminobisphosphonates (NBP); Gly₄Ser (GS).



HIS mice have been created with the aim to bridge the gap between small animal models and human studies and to explore human immunity in an *in vivo* experimental system. Several advances over the last years have improved the overall human engraftment and the development of various lineages of blood cells in HIS mice. For example, while B and T lymphocytes are quite well developed in HIS mice (albeit with some maturation deficits), both myeloid and lymphoid innate cells are underrepresented. Their low frequency may be the result of both the incapability of progenitor cells to further differentiate or migrate from primary organs and/or the lack of survival and proliferation signals in mature cells. Overall, we understand very little about what drives development of a particular blood cell type. At the same time, the deficiencies themselves found in these models provide hints of the molecular cues necessary for normal balanced hematopoiesis³⁶⁵.

During hematopoiesis a steady state is established between HSC self-renewal and lineage differentiation that is maintained by regulatory signals (cell interactions, soluble factors) coming from cells within the environment. External cues also help maintain the survival and functionality of mature hematopoietic cells throughout their lifetime. In HIS mice, many cells present within hematopoietic niches (osteoclasts, vascular cells, perivascular cells) are of murine origin^{366,367}. Their products (chemokines, cytokines and growth factors, etc.) may act in a species-specific fashion. In some cases, they will be sufficiently divergent in their structure from their human counterpart that they do not function on human cells. Several approaches in HIS mice have been devised to address this issue (replacement of mouse genes with human counterparts, exogenous deliver the human molecules *in vivo*, etc ^{260,261,277,368}). These optimized HIS models show a 'balanced' development of most hematopoietic lineages thereby more closely resembling the human condition.

The Flt3-deficient BRGS HIS mouse model developed in our lab efficiently reduces the generation of mouse DC subsets thus providing an open 'niche' for human DCs. Using exogenous Flt3L allow for a selective boosting of several myeloid populations, including CD141⁺DCs, CD1c⁺DCs, pDCs and CD14⁺ monocytes at a systemic level with the BM showing the most pronounced effect. CD135+ myeloid precursors were increased in this model. Previous reports had revealed a prominent effect of Flt3L in the *in vitro* and *in vivo* development of NK cells. Interestingly, our HIS model not only confirmed the effect on NK cells but it also showed, for the first time, an increase in the frequency and total numbers of human lineage⁻CD127⁺ helper ILCs. The manuscript "A functional DC crosstalk promotes human ILC homeostasis *in vivo*" analyzed the phenotypic and functional characteristics of each ILC subset identified in this novel HIS mouse model.

i. What is the mechanism underlying the ILC boost in BRGSF HIS mice?

Given the bidirectional crosstalk between DCs and NK cells both in homeostasis and during the immune response, the increased DC compartment in BRGSF mice was predicted to impact human NK cell development. The primary cytokine governing NK cell differentiation, proliferation and survival is IL-15. When bound to its high affinity IL-15R α , soluble or membrane-bound complexes are formed that can then act on IL-2 $\beta\gamma$ R bearing target cells (so called "trans-presentation") triggering downstream signaling^{302,369}.

NK cell proliferation and survival is also regulated by IL-2³⁷⁰. CD4⁺ T cells are the main IL-2 producers but IL-15 can also boost human T cell homeostasis ³⁷¹, suggesting that boosted BRGSF might have an enriched IL-2 milieu as a consequence of the higher IL-15 availability. One could expect then that CD56^{bright} cells would overtake in number the CD56^{dim} subset due to its higher expression density and hyper-responsiveness to IL-2³⁷². Nonetheless, the ratios of the two populations remain stable in reconstituted BRGSF mice after Flt3L treatment. This may be due to the timing of T cell development in the model (T cells develop later after HSC engraftment) thus limiting the environmental concentration of human IL-2 in the periphery.

While IL-15 is fundamental for NK cell development, helper ILCs are dependent on the cytokine IL-7. Murine IL-7 has been shown to have some inter-species activity based on its capacity to trigger the same signaling pathways than hIL-7 in human T cells³⁷³. However, the widespread expression of IL-7 receptor in the neighboring developing human lymphocyte precursors and mature cells ($\gamma\delta$ -T, NKT, $\alpha\beta$ -T and B cells) limits the availability of the cytokine. Although the main source of IL-7 are the stromal and vascular endothelial cells, DCs have also been shown to produce it at low levels in steady-state conditions and 4-fold stronger after CD40L/IFN γ activation³⁷⁴. Given the fundamental role of IL-7 in the development of CD7⁺CD127⁺ ILC cells, this is likely behind the positive effect observed in the total cell number in BRGSF after the myeloid boost.

Besides the direct effect of DC-derived cytokines on ILC proliferation and survival, a second mechanism involving a direct effect of Flt3L in Flt3-expressing ILC progenitors

might also operate and foster their development. Both NK cells and hILCs differentiate from HSC via the CLP that has the potency to give rise to all innate and adaptive lymphocytes. While the reported ILC precursors in mouse and human, including the ILCP we identify here (Paper II) is negative for Flt3, Renoux and colleagues have recently found a NK-restricted progenitor (NKP, Lin⁻CD34⁺CD38⁺CD45RA⁺CD7⁺CD10⁺) population that partially (50%) express the receptor of Flt3L. NKP is downstream of CLP, that expresses it in 70% of the cells, and give rise to NK cell but does not have T, B, myeloid or helper innate lymphoid cell potential¹³⁶. Given the expression of Flt3 in both CLP and NKP and maybe other yet-unknown ILCPs, it is likely that Flt3L is directly signaling on this precursor populations and this might account for the ILC enhancement we observe.

ii. Why do ILCs have enhanced function in BRGSF HIS mice?

All ILC populations detected in BRGSF HIS mice demonstrated an increased functional competency after Flt3L treatment. We postulate that the mechanism underlying this increased function is probably linked to the boosted myeloid compartment that provides an enriched niche with higher cytokine availability. It has been shown in several mouse models that NK-cell preactivation by DCs is required for an efficient immune response against viral infections and tumors^{375,376}. Further to the transpresentation of IL-15 that promotes not only proliferation and survival of NK cells but also prime protective responses, DCs are capable of producing a myriad of other key factors. For instance, IL-12 production, particularly by infDCs, efficiently stimulates IFNγ secretion by NK cells, and this is greatly enhanced after microbial-mediated activation of TLRs in DCs. When IL-18 is present, the expression of IL-12 receptor is upregulated rendering the NK cells more responsive. This synergy between IL-18 and IL-12 enhance, in turn, NK cell cytolytic activity³⁷⁷. The release of type I IFNs by pDCs and CD141⁺ cDCs also stimulates NK cell cytotoxicity particularly in the context of viral infection and provoke the upregulation of stimulatory receptors such as CD69 ³⁷⁸.

The presence of functional mature DCs in our model accompanied by the increased of ILCs creates an ideal system that is susceptible to viral infections thus providing a platform to observe the very important interplay between those two families. Indeed, the presence of viral dsRNA triggers TLR3 activation both in NK cells and in DCs that engage in a crosstalk that promote IL-12 mediated NK activation and the selective survival of mature DCs (NK cell-

mediated "editing" of DCs) and the eventual induction of Th1 responses. Mouse models of cytomegalovirus showed that NK cells control the frequency and length of viral antigen presentation by DCs ^{379,380}, highlighting the central immunoregulatory role of NK cells in CMV infection. In humans NKG2D and NKp46, which are expressed by BRGSF mice where found to be involved in the recognition of influenza-infected DCs triggering the activation and immune response³⁸¹.

The phenotypic similarities between NK cells in HIS mice and in normal human tissues suggest that HIS mice provide a relevant model to study NK receptors *in vivo* under certain conditions. This could be especially interesting in the context of infectious diseases, for example during the expansion of CD56⁺NKG2C^{bright} NK cells in response to HCMV⁹⁸.

The role of DCs and NK cells in viral infections has been already explored in a few reports involving humanized mice. For example, NRG mice disclosed an opposing role of pDCs in HIV-I replication and promotion of the immunopathogenesis that depends on IFN-I providing hits for novel therapeutic strategies ^{382,383}.

Other factors, such as chemokines serve as mediators in the DC-NK interplay. This is the case of CX3CL1, which is a soluble and membrane-bound chemokine expressed by DCs and up-regulated upon maturation. NK cells express CX3CR1 and its activation triggers an IFN γ response³⁸⁴.

In the same way, ILC2 and ILC3 responses are enhanced in the presence of the enriched cytokine environment created in our model. The range of cytokines produced by DCs includes IL-1b, IL-4, IL-6, IL-21, IL-23 and IL-33, which have various activating roles both in Th cells and in ILCs as discussed earlier in this manuscript^{385,386}. In our Flt3L boosted model, both the basal expression and the enhanced release of these cytokines upon DC activation provide stimulatory signals that activate ILCs more efficiently than the non-boosted system.

iii. How do ILC develop in BRGS-based HIS mice?

Subsets of ILCs develop during the fetal period (particularly the LTi cells that promote organogenesis of lymphoid structures) and which then persist during adult life^{133,186}. While the fetal development is absent in the humanized mice setting, leading to the lack of LTi cells and likely other subsets of ILC3s³⁸⁷, post-natal ILC development could potentially follow the same process than in the human system. The presence of adaptive lymphocytes

and NK cells proves that CLPs in HIS mice are capable of giving rise to lymphoid progeny. In mouse studies, the differentiation of CLPs into mature ILCs seems to follow the same scheme with progenitor populations existing in both embryos and adults. However, parabiosis studies in mice revealed that the replacement of tissue helper ILCs is minimal during adulthood, in contrast to NK cells¹⁴⁴. Since human engraftment takes place after-birth in HIS mice, that would explain the better reconstitution of NK cells in comparison to ILCs and would be a major limitation for the latter. Therefore, only "adult" ILC development would take place, thus lessening the ILC heterogeneity. Nevertheless, it is not known whether also potential neonatal-developed ILC progenitors can contribute to the adult ILC pool and maybe compensate for the fetal deficiency. In that case, the post-birth timing of the human reconstitution would be critical on the progeny potential of the precursors and should be taken into account.

ILC diversification is regulated by the upregulation of signature transcription factors but the environmental signals that drive that regulate this process are poorly understood. Several TFs are known to be involved in the development of NK cells while hILC1 development program is less well appreciated, as reviewed elsewhere ^{133,388}. The common trait of type I ILCs is the capacity to produce IFN γ , which is regulated by the transcription factor T-BET. It is possible that the signals driving T-BET expression are linked to ILC1 commitment, although there is not clear evidence to support this hypothesis. T-bet is induced in two waves: the first comes after TCR or IFN γ -STAT1 signaling and the second by IL-12 mediated activation of STAT4. Later on, the IFN γ produced by the type I cell maintains Tbet expression in a positive feedback loop ³⁸⁹. HIS mice could potentially support that process since the T-BET inducers, IFN γ -STAT1 and IL-12-STAT4, can be found in humanized mice. Nevertheless, whether this signaling occurs in an appropriate spatio-temporal setting that favors ILC development has not been addressed.

ILC2 specification and function depend on two transcription factors, ROR α and Gata-3. Transient Notch signal has also been shown to have a role in that process in CLP cultures and this has been further suggested *in vivo* since ILC2Ps expanded upon signaling through Notch2 ³⁹⁰. Given that OP9 cell lines ectopically expressing Notch ligands can drive both mouse and human T cell development ³⁹¹, the observation of ILC2s in BRGSF mice would imply interspecies Notch signaling also *in vivo* in humanized mice. On the other hand, although mainly secreted by necrotic cells after tissue injury, IL-33 can also be produced by DCs at low levels, being further enhanced by DC stimulators such as LPS or PGE₂. As suggested in the literature, the synergy between Notch, IL-7 and IL-33 after Flt3L could drive the increased development of the Gata-3 ILC2s observed in BRGSF mice.

It is well appreciated that RORyt is the TF designating mouse ILC3 commitment and recently it has been, as well, supported in humans after the identification RORyt⁺CD34⁺ cells as lineage-specific progenitors of ILC3 that are enriched in tonsils and intestinal LP³⁹². However, the environmental cues that drive the upregulation of RORyt are not as clear. Both in the fetal and the adult development of ILC3 Notch signaling seems to be involved, at least partially, and this signaling is dependent on the TF aryl hydrocarbon receptor (AHR). In mice, ILC3 development is indirectly regulated by the intestinal microbiota and the derived xenobiotic substances are sensed by AHR³⁹³. Deficiencies of Ahr^{-/-} mice in the development of a number of cell lineages including several T cell subsets and also of ILC3 confirmed its indispensability. Given the notable presence of ILC3 in our model these interactions might be taking place in the mouse or otherwise alternative pathways may give rise to human ILC3s. It would be interesting to examine RORyt-expressing precursor populations and to assess their progeny potential *in vivo* as well as the dependency on Notch, AHR and different types of microbiota^{133,394}. Furthermore, BRGSF mice can provide the opportunity to study tissue specific properties of those precursors and also the developmental relations between different ILC subsets.

iv. Are HIS mice relevant for human lymphocyte development discovery?

While mouse ILC development has been dissected in diverse ILC precursor stages ^{133,395}, the human hematopoietic hierarchy is less well characterized. Downstream of CLP only the NKP, mentioned earlier, has been described both *in vitro* and *in vivo* giving rise only to cytotoxic NK cells but not other hILCs³⁹⁶. Regarding hILCs, several reports have identified putative human progenitors committed to a specific ILC group but any has provided evidence of an *in vivo* ILC common precursor ^{392,397,398}. The study of mature ILC populations described in the first part of this thesis set a precedent for the further exploration of human precursor populations in our model.

Human peripheral blood studies conducted in the laboratory identified a CD117⁺CD127⁺ ILC-like population that does not express signature transcription factors and is not capable of producing cytokines, but can give rise to mature ILCs after in vitro culture. Analysis of central and peripheral organs of reconstituted HIS mice gave rise to the observation of a similar cell population. These Lin⁻CD7⁺CD127⁺CD117⁺ cells are present in

BM, spleen, lung and liver and, similarly to the human counterpart; do not express EOMES, T-BET, ROR γ t or GATA-3 at the level of the mature ILCs. Furthermore, while no cytokine production was observed *ex vivo*, when sorted and cultured with IL-2, IL-7, IL-1 β and IL-23, these cells expanded and acquired the capacity to produced IFN- γ , IL-13, IL-22 and IL-17 in different proportions depending on the mouse. The characterization of an ILC precursor population in both the human system and the HIS mice provides an excellent example of the power of HIS models to recapitulate human immunobiology. Although we did not have access to BM tissues, the presence of ILCP in humanized mice BM suggests that this is the case also in humans. In that case, ILCP would account for an ILC precursor that arises from the BM and patrol through the circulation the peripheral tissues providing an *in situ* source of ILCs upon infection or inflammation. Further studies in humanized mice may help shed light on the mechanisms that regulate ILCP responsiveness within different tissue environments.

v. Immunotherapeutic implications: beyond the NK cells

The enriched ILC development in Flt3L-boosted BRGSF mice and their relevant tissue distribution and functionality opens a wide range of possibilities for studying the steady-state development and biology and the role of this cell family in immunosurveillance and anti-viral responses.

Based on its phenotypic resemblance and the capacity to produce type I cytokines it has been assumed that ILC1 may be involved in Th1 responses similar to cytokine producer NK cells. However, their actual contribution to inflammation has not been fully explored. As with NK cells, murine studies have revealed an important role for ILC1 in mediating immunity against intracellular pathogens like T. Gondii ³⁹⁹ and in tumor immunosurveillance ⁴⁰⁰ but few studies have been reported yet in any of these diseases in the human system. Only a positive correlation has been reported in non small-cell lung tumors between the intratumoral type 3 ILC numbers and the clinical outcome¹⁹⁵. Despite this protective role, IL-23 has been detected in various human cancers, and increased IL-17 and IL-23 expression correlated with severity of the disease and worse prognosis. Human tumor models are needed to dissect the role of ILCs in tumorogenesis and study the impact of its modulation as a therapeutic approach.

In humans, ILC1s are increased in patients with chronic hepatitis B infection and they are associated with the hepatic damage ⁴⁰¹. Many reports have demonstrated that ILCs are

mostly tissue resident and only at later time points of the infection tissues get replenish from circulating ILCs or ILC precursors ¹⁴⁴. However, usually only ILCs in peripheral blood can be studied humans while the HBV humanized mouse models could provide further understanding of the involvement of ILC1 in hepatitis infection. Mouse models harboring both the immune system and human hepatocytes have been created that support the natural course of acute HBV infection and chronic hepatitis ^{402–404}. Combination of these systems with the BRGSF model we present here could provide a better understanding of the virus pathogenesis and its interaction with the innate immune system.

There are limited data on the role of ILCs in other human infectious diseases. Recently a humanized NSG mouse model showed reduced ILC3 numbers, as observed in chronic HIV patients, and suggested a mechanism dependent on pDCs for that cell reduction, which was reserved after antiretroviral therapy ³⁸³. Strikingly, filarial worm infection in human provoked an expansion of type 2 ILCs suggesting a role of these cells in immune defense as previously demonstrated in mice ¹⁷⁸. Any HIS model has been developed to our knowledge that could shed light on the protective role of ILC2 in worm infection so far.

IFNy producing CD127⁺ ILC1s have been identified in human gut and they are increased in patients of Crohn's disease. The induction of acute or chronic inflammatory bowel disease (IBD) using chemicals such as dextran sodium sulfate (DSS) could be achieved in BRGSF mice recapitulating the observations in human. In this line, a humanized NSG model of IBD has revealed that the increase in ILC1s is a direct consequence of acute inflammation and not due to a long-term ongoing inflammatory state¹⁴⁷. Intestinal inflammation is also associated with type 3 ILCs since their phenotypes are altered in patients with IBD with selective enrichment in inflamed tissues. Their potent capacity to produce IL-17A and IL-22 greatly contribute to the inflammation given that ILC depletion or blockage of those cytokines abrogates colitis in mice, although the depletion strategy used in that study was not specific for ILC3¹⁴⁸. Nevertheless, this pathogenic role in chronic intestinal inflammation needs to be confirmed in human IBD models. Strikingly, mouse IL-22 producing ILC3s are necessary for fighting acute bacterial infection since IL-22 blockade exacerbated the associated inflammation likely resembling the situation in human E. Coli infections. Furthermore, IL-22 producing cells are necessary for tissue repair particularly in the context of IBD rising more questions on the potential role of this ILC subset in human gut immunity⁴⁰⁵.

ILC2 play a pathogenic role in many inflammatory respiratory diseases as well as in viral related diseases in the mouse, but their phenotype and distribution differ from humans. In humans, higher proportions of ILC2s have been observed in patients of chronic rhinosinusitis and allergic asthma while the tendency is inversed for chronic obstructive pulmonary disease, where both lung tissues and PBMCs contain less ILC2s than in healthy individuals. The mechanistic explanations for those variations are largely unknown highlighting the need for better models that reconstitute human ILC2s and myeloid cells, particularly granulocytes and mast cells, which are the main responsible for mediating allergic responses. Such a model could be the NOG IL-3/GM-Tg with contain human IL-3 and GM-CSF as transgenes leading to the enhancement of the myeloid compartment, particularly basophils and mast cells ³⁶⁴. This HIS mouse model recapitulated some of the events occurring in asthmatic inflammation upon administration of IL-33 and should provide an opportunity to study the involvement of ILC2 in the pathology.

ILCs sense signal cytokines shed by myeloid and non-hematopoietic cells and respond with the production of effector cytokines that active local innate and adaptive effector function thus behaving as integrators of the immune response. Rapidly after infection or stress ILC produce type 1, type 2 and type 3 cytokines that shape the adaptive response through expression of MHC class II molecules and regulation of DCs⁴⁰⁶. The presence of ILCs, T cells and DCs in humanized mice offer a means to better understand the crosstalks among the three-effector cells in a tissue specific manner in homeostasis and in the context of inflammation.

CONCLUDING REMARKS

In a context in which the divergence between mouse and human immune system is increasingly appreciated, my thesis aimed at developing a humanized mouse model that allows researchers to better understand human innate immunity and to bridge the gap with the clinic. For the first time, we describe a humanized mouse model that supports the development of all three ILC subsets at high levels and functionally competent in steady state. Furthermore, we show that humanized mice are capable of providing valuable information about unknown developmental pathways in human hematopoiesis.

One challenge of ILC research is to understand how these cells interact with other innate, adaptive and non-hematopoietic cells to resolve inflammation. Over the last decades others and we have set up disease models easily applicable to our BRGSF system or others designed to address specific questions of the immune response. These "next generation" HIS-pathology models, that could be patient-personalized, have the potential to uncover new aspects of ILC biology and to offer a platform to manipulate ILC responses thus providing immune protection.

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Humanized Mice for HIV Research



Chapter 14: NK cell differentiation in human immune system (HIS) mice

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14.1. Introduction

Natural Killer (NK) cells were initially identified by their ability to spontaneously lyse tumor cells without prior activation, and early efforts focused on understanding the mechanisms controlling NK cell cytotoxic activity. An equally important issue was why and how 'natural killers' did not attack self-tissues (NK cell 'tolerance'). It was later learned that NK cells use germ line-encoded inhibitory NK cell receptors that distinguish self-MHC from 'altered', 'stressed' or non-self-MHC molecules expressed at the cell surface. Thus, NK cells and adaptive lymphocytes (B, T) use fundamentally different strategies to detect invading pathogens, with NK cells surveying self MHC for alterations (stress, transformation), as opposed to B and T lymphocytes that detect foreign pathogens or pathogen-derived peptides, respectively.

Infection often leads to changes in the expression of cell surface molecules. This indirect 'signature' can be 'read' by lymphocytes that patrol tissues. Many viruses down-modulate MHC class I expression on the cells they infect, and as such, become targets for NK cells that survey self-MHC molecules at the cell surface. The essential role of NK cells in anti-viral defense is demonstrated by the increased susceptibility to infections (particularly herpes and papillomavirus) observed in NK cell-deficient patients (1). Moreover, some viruses have evolved mechanisms that allow their potential escape from NK cell recognition. These include virus encoded proteins that complex and inhibit ligands for NK cell activating receptors, as well as proteins that interfere with soluble factors required for the inflammatory response. In contrast, cellular transformation does not necessarily lead to the reduced expression of MHC class I molecules but is more generally associated with the inducible expression of 'stress' proteins. Ligands for NK cell activating receptors (including NKG2D or natural-killer group 2, member D expressed by all NK cells) represent another detection system that operates independently of the inhibitory MHC-specific NK cell receptors.

Only a fraction of peripheral NK cells (in mice or man) truly act as 'natural killers', while most NK cells possess additional effector molecules that allow them to modify cells within their microenvironment. These include soluble cytokines and chemokines that can amplify immune recognition via up-regulation of MHC molecules on target cells (IFN-g), activate neutrophils (TNF-a), recruit hematopoietic cells (GM-CSF) and potentially suppress the inflammatory response (IL-10). Thus, NK cells are involved in different steps of the immune response (detection, first-line defense, and subsequent amplification), they interface directly with cells involved in the adaptive immune responses (dendritic cell cross-talk), and they are potentially involved in the regulation of autoimmunity. Diverse NK cell

functions can be modulated and in some cases evolve to provide new biological effectors depending on environmental signals.

In the present chapter, we briefly review the main features of NK cell development and function before summarizing knowledge on the interactions between NK cells with HIV. We will then turn to a review of the most recent advances regarding NK reconstitution in HIS mice. We will conclude by trying to give the reader an idea of the current status and potential of HIS mice for deciphering NK cell-mediated response in HIV infection.

14.2. NK cells in mice and man

14.2.1 NK cell development: a step-wise transcriptional process that generates diverse functional subsets

Several mature NK cell subsets have been described that demonstrate unique tissue localizations, as well as phenotypic and functional differences. We will not review this subject that has been extensively reported (2, 3), but will only remind the readers that the origins of this diversification remain poorly understood. Several mechanisms are likely involved, certainly those controlling tissue-specific homing, but also the presence of unique environmental cues that condition the activation states of tissue-resident NK cell subsets (4).

The development of fully differentiated NK cells from pluripotent hematopoietic stem cells (HSCs) is controlled by the integrated influence of cytokines and growth-regulatory molecules that promote proliferation together with transcription factors that activate or repress lineage-specific gene expression (5). The result is a stepwise transformation that is accompanied by an evolving pattern of receptors whose integrated and coordinated signal control and regulate the acquisition of the functional response (6). Three main stages of maturing NK cells can be recognized: NK cell precursors (NKP) that are committed to the NK cell lineage, immature NK (iNK) cells that are acquiring phenotypic and functional competence, and mature NK (mNK) cells that have completed the developmental process.

The process by which a hematopoietic stem cell (HSC) becomes a committed NKP is perhaps the most poorly characterized in terms of receptor expression and transcriptional activity. Recently, this stage has been refined with the use of reporter mice and additional cell surface markers that define developmental intermediates between the common lymphoid progenitor (CLP) and a 'refined' NKP (7, 8). Similarly, human NK-lineage precursors have been isolated from several tissues including BM, peripheral and umbilical cord blood, thymus, secondary lymphoid organs, liver and decidua (9-12). NKP are functionally defined in either species have potential to give rise to NK cells but no other

hematopoietic lineages. While this appears to be satisfied in the mouse (7, 8, 13), human pro-NK (CD34⁺CD38⁺CD45RA⁺CD7⁺) and pre-NK (CD34⁺CD45RA⁺CD117⁺CD94⁻CD122⁺) subsets retain some multi-lineage potential suggesting that these are not homogeneous populations of human NKP (14, 15).

The transcriptional activity that restricts NKP potential to the NK cell lineage is not fully understood. The inhibitor of DNA binding 2 (Id2) is critical for NK development by preventing the transcriptional activity of E box TFs (16). Still, this is not sufficient to ensure NK cell lineage development, since Id2 is also required to generate distinct innate lymphoid cell (ILC) subsets from CLP (17). Presumably a combination of TFs are involved that may include Tox, T-bet (Tbx21) and Eomesodermin (Eomes) (18, 19). Recently, Ets1 has emerged as an early regulator of NK precursor development controlling Id2 and Tbx21 expression (20). It is perhaps through combinatorial TF expression that the NK lineage diverges from CLP. This initial TF profile may also encode cytokine receptors (including the IL-2/IL-15 receptor β chain CD122) required for IL15 responsiveness. Once these precursors encounter a suitable IL-15-containing environment, the NK lineage potential may be reinforced, sealing NK commitment.

The transition from NKP to iNK cells generates cells with partially mature NK phenotypes, at the cell surface (ie: with some but not all NK cell receptors) and with respect to effector cell functions. In the mouse, this stage is marked by acquisition of NK1.1 and NKp46, while in human NK cells this involves up-regulation of CD56 and NKp46 expression (4). Several TF are expressed in iNK cells, including the nuclear factor induced by IL-3 (Nfil3, also known as E4-binding protein 4, E4bp4) (21-23) and the T-box factor Tox (24). Little is known how these TF control NK cell maturation in general, or the selective NK cell receptor expression profiles of iNK cells in particular. Concerning effector functions, most immature NK cells are not cytotoxic and do not secrete IFN-g, although some reports have proposed that iNK cells have distinct cytokine production profiles (25, 26). Gata3 plays a role in NK cell differentiation at this stage by regulating cytokine production (27). Interestingly, Nfil3/E4bp4 has been proposed to regulate Gata3 (23), providing a potential link between TF that control iNK cell maturation.

The terminal differentiation of mNK cells is marked by the acquisition of DX5/CD49b in mice, whereas in humans this stage is achieved with the generation of CD56^{hi}CD16⁻ and CD56⁺CD16⁺ NK cell subsets. In both species, mature NK cells express receptors of the CD94/NKG2C family and also variable and stochastic expression of MHC class I-specific NK cell receptors (KIR in human, Ly49 in mouse). Many of these inhibitory receptors are involved in sensing target cell MHC expression (that indirectly measures cell 'health') and are critical regulators of NK cell maturation in the bone marrow and activation in the periphery. The mechanisms behind the 'education' process that generates KIR/Ly49

repertoires remains one of the greatest unsolved enigmas in NK cell biology. Other surface markers increase expression on mature NK cells compared to iNK, including CD11a, CD11b, CD16 and CD43. Some of these molecules are involved in cell adhesion and may be critical for allowing NK cell to eliminate target cells. The functional capacity of NK cells also increases as cells progress to mNK: perforin, granzymes, cytokines (IFN-γ, TNFs). These phenotypic and functional changes occur in parallel with shifting TF profiles: expression of Blimp1, Eomes, Tbx21, MITF, MEF and CEBP-g are up-regulated at this stage and deficiencies in these factors compromise NK cell effector functions. While not all TF/target gene associations are completely understood, both Blimp1 and Tbx21 appear to directly regulate cytokine, perforin and granzymes B production (18) (28).

14.2.2. Roles for cytokines in NK cell development and function

NK cell differentiation is regulated by several distinct cytokines among which the common gamma chain-dependent cytokines have a decisive role. In particular, IL-2, IL-7, IL-15 and IL-21 display specific and overlapping roles in the generation and maintenance of NK cell and may have a differential impact on the development of specific NK subsets (29). Studies have shown that CD56hiCD16- NK cells in man express the high affinity trimeric IL-2Rabg receptor and are exquisitely sensitive to low levels of IL-2, where as CD56⁺CD16⁺ NK cells in man and all NK cells in mice express the intermediate affinity IL-2Rbg complex and can only be triggered by higher (non-physiological) levels of IL-2 (30-32). Moreover, mice or humans deficient for IL-2 or IL-2Ra do not have apparent defect in peripheral NK cell reconstitution (33-35). These studies indicate that IL-2 may have a dose-dependent effect on the maturation and function of peripheral NK subtypes, but does not play a role in mainstream NK cell development in either species. IL-15 exerts its effects via 'transpresentation' by soluble IL-15R α or through membrane bound IL-15R α to cells expressing the IL-2Rbg complex (36-43). In contrast to IL-2, mice deficient for IL-15 or IL-15Rα have defects in NK population, which confirms the primary role of IL-15 for the development and homeostasis of NK cells (44, 45). Both cytokines are known to stimulate the proliferation, survival and activation of NK cells and have broad impact on other lymphoid cells. Abundant granzyme B and perforin can be produced from pre-stored mRNA in NK cells after stimulation of IL-2 or IL-15 to increase NK cell mediated cytolytic activity (46, 47).

Unlike IL-2 and IL-15, IL-7R α is not expressed by most circulating NK cells. However, a subset of NK1.1⁺ cells in murine thymus expresses IL-7Ra as do the human CD56^{hi}CD16⁻ subset (48, 49). Mouse CD127⁺ NK cells distinguish themselves from classical NK cells by their dependency on IL-7 and GATA-3 and display high cytokine secretion potential but low cytolytic activity. Whether IL-7 can boost the homeostasis of NK cell subsets in vivo remains to be tested. IL-21 primarily produced by activated CD4 T cells and plays several roles in immune responses, primarily by inducing the functional maturation of B, T and NK cells. Although IL-21 is not required for mainstream NK cell development, this cytokine can co-stimulate NK cells (with IL-15 and IL-12) to proliferate and potentiate their ADCC activity (50-52).

Apart from common gamma chain dependent cytokines, many other cytokines can function to regulate mature NK cell responses. For example, IFN- α/β is known to induce cytolytic activity of NK cell during viral infections, IL-12 and IL-18 are potent stimulators of IFN-a secretion, and IL-27 can promote 'regulatory' activity (IL-10) from human NK cells (53). In addition, negative regulators of NK cell function likely include IL-10, TGF-b and in some cases, type I interferons (54).

14.2.3. Mechanisms of NK-cell mediated responses

The major effector functions of NK cells (cytotoxicity and cytokine secretion) are controlled through an integration of signals that arise from an array of activating and inhibiting receptors and the presence of pro-inflammatory cytokines. The inhibitory receptors, encoded by the killer Ig-like receptor (KIR) multi-gene family in humans and the C-type lectin-like genes family (Ly49) in mice, are responsible for the MHC 'self-tolerance' through recognition of host MHC class I molecules (HLA in humans) (55). While this education process is poorly understood, it has been shown that potentially 'autoreactive' NK cells become functionally hyporesponsive during this process (56). This therefore selects for NK cells that react to targets cells that lack normal expression of MHC-self molecules (due to viral infection or malignant transformation), the so-called 'missing-self' hypothesis (57). Loss of inhibition then allows the subsequent activation of the NK cells in immune responses. Rarely the situation is 'black and white' with complete loss of MHC expression. More often, it is the balance between activation and inhibition that determines the outcome. For example, when a NK cell recognizes a stress- or infection-induced ligand via an activating receptor (such as CD16, NKG2D or natural cytotoxicity receptors NKp30, NKp44 and NKp46) in a low MHC-I expression setting, the NK cell becomes activated and the "killing machinery" is engaged. This involves the exocytosis of cytotoxic granules containing perforin and granzymes via an immunological synapse. Additionally, small peptides and the pro-apoptotic molecules Fas ligand and TRAIL (TNF-related apoptosisinducing ligand) may also be delivered. These induce a death-inducing signaling complex that leads to the induction of caspase-mediated apoptosis (58, 59).

NK cells bear the CD16 receptor (FcγRIIIa) for IgG and can mediate antibodydependent cellular cytotoxicity (ADCC) especially against antibody-coated tumor cells. CD16 engagement triggers NK cell activation, cytokine production and degranulation that leads to target cell lysis. Some HIV-specific peptides have been proposed as possible ADCC targets for a NK cell-based vaccine, as reviewed below, as well as, epitopes unregulated during tumorigenesis, such CD20, Her-2/neu, epidermal growth factor receptor, vascular endothelial cell growth factor, just to mention a few (60). The potential of this approach is strongly dependent on FcR alleles and HLA/KIR-mediated NK cell education (61).

Human and mouse NK cells are responsive to soluble factors, such as IL-12 and IL-18, that lead to robust secretion of IFN- γ , TNF- α , granulocyte macrophage-colonystimulating factor (GM-CSF), IL-10, and IL-13 (62). Some subsets of NK cells appear more responsive in this respect (CD56^{hi}CD16⁻ human NK cells, CD127⁺ mouse NK cells), but it should be stressed that production of certain cytokines (for example IFN-g) is a general property of all NK cells. The cytokines produced by NK cells may depend on the combination of monokines present within the environment. The cytokine-mediated activation of NK cells shapes the subsequent recruitment and function of other hematopoietic cells. In the case of anti-viral T-cell responses, NK cell activation regulates CD4 T cell-mediated support for the CD8 T cell control of viral pathogenesis, while during infections NK cells shape recruitment and activation of myeloid cells (63, 64).

Several observations from in vitro experiments and studies with mouse models as well as with tumor and transplantation patients confirmed the regulatory effect of Treg cells on NK cell activity. Treg cells inhibit NK cell proliferation, cytotoxicity and IFN- γ secretion under certain conditions through a TGF β -mediated interaction (65) that may be counteracted by the accumulation of IL-15R α expressing DCs, or, as claimed in recent works, by a IL-2-dependent signaling. This mechanism implies a CD4⁺ T cell secretion of IL-2 that is taken up by Treg cells, thus preventing NK cell stimulation (66). Another possible player in the crosstalk network around NK cells are neutrophils as reported recently based on inflammation in vitro experiments. It remains unknown, however, to which extent these two cell types cross-regulate activities in vivo (67).

Over the last years researchers have speculated with possibility that NK cells might display immunological memory, a trait that has historically characterized adaptive immunity. A number of viral antigens, including HIV-1, elicit recall responses from hepatic mouse NK cells by a mechanism dependent of the chemokine receptor CXCR6 (68). Moreover, MCMV infection generates a population of NK cells with heightened effectors functions that are reminiscent of memory T cells (69). Whether 'memory' NK cells exist in other disease states or have an important role in human immune responses remains to be determined.

14.3. NK cells in HIV infection

14.3.1. NK cell-mediated control of HIV infection

NK cell-mediated innate immunity appears to play a pivotal role in restraining HIV-1 viral replication at earlier stages of infection as demonstrated by the correlation between the levels of viremia observed in patients and the expression of specific combinations of KIR-MHC class I alleles (70). Moreover, increased NK-cell mediated responses have been documented in sero-negative exposed individuals compared to healthy and HIV-1-positive patients (71). In general, an increase in CD56⁺CD16⁺ subset and a reduction in CD56^hCD16⁻ NK cells are observed early in HIV-1 infection. As the infection becomes chronic, Siglec-7 expression on NK cells increases, followed by the emergence of a population of functionally anergic NK cells that are CD56⁻ and present increased expression of inhibitory receptors (72, 73). These observations suggest that 'exhausted' NK cell phenotypes can result from excessive activation in the context of prolonged virus replication. In some studies, CCR5 expression on NK cells is also upregulated in viremic patients (74) that could indicate hyper-activation. In vitro, exposure of NK cells to HIV envelope proteins can induce some of these changes in gene expression (75, 76).

The putative mechanisms that NK cells use to restrict HIV replication are not fully eludicated. Both cytolytic and secretory mechanisms are possible, including granulemediated lysis of virally infected target cells and/or production of soluble factors that limit viral spread. Along the latter, NK cells can produce CC-chemokine ligand 3 (CCL3), CCL4 and CCL5, all of which are ligands for CCR5 co-receptor required for HIV entry (77).

For many years, a focus of research has been the identification of viral proteins that could be targets for elimination of virally infected cells by antibody-mediated cellular cytotoxicity (ADCC). It has become now clear that directing ADCC responses toward particular HIV epitopes may be more efficient for controlling viremia (78). However, the wide variety of viral strains complicates the identification of a conserved epitopes capable of triggering a robust and broadly reactive ADCC response. Some potential vaccine targets have been proposed, including Vpu (a multifunctional protein from HIV-1 that can be found on the surface of infected cells) or the Env gp120 epitope defined by mAb A32 (79, 80).

Epidemiological studies have delved into the importance of KIR-HLA interactions in the NK cell-mediated control of HIV, which is already extensively appreciated for T-cell responses, however, there is not yet complete consensus regarding the specific allele variants associated with better HIV-1 disease outcomes (81).

14.3.2. Viral evasion and modulation of NK cell phenotype and function in HIV infected patients

NK cell function in HIV infected patients has been shown to be defective in many respects. One direct mechanism by which HIV can directly modulate NK function is through its viral proteins. HIV protein Tat inhibits NK mediated cytolysis by blocking calcium channel activity. This in turn inhibits the cytolytic capacity of NK cells through two distinct mechanisms: reduced degranulation and inhibiton of FasL upregulating (82, 83). Another example concerns the HIV envelope protein gp120. Binding of gp120 to CXCR4, constitutively expressed on NK cells, can induce autophagy leading to NK cell death (84).

While many viruses down-regulate MHC class I molecule during infection, HIV infected cells retain relatively normal levels of MHC class I molecules. Instead, HIV infection, via the viral protein Nef, selectively reduces expression of HLA-A and HLA-B that are mainly used to present viral peptides to cytotoxic T cells. In contrast, Nef does not modify expression of HLA-C and HLA-E molecules that serve as ligands for inhibitory NK cell receptors (85-87). In addition, Nef also binds and degrades MICA and ULBPs in HIV infected cells, which are ligands for the NK cell activating receptor NKG2D (88). Nolting et al reported that chronic HIV-1 infection is associated with a specific defect of NKG2D-mediated NK cell activation, due to elevated levels of the soluble (shed) MICA that are released by HIV-1 infected CD4⁺ T cells into patient sera. Increased serum MICA results in NKG2D down-regulation and a profound dysregulation of NK cells effector functions (89). Hence, by differentially modulation of HLA molecules and NKG2D ligands on infected cells, HIV escapes from most antiviral activities mediated by CD8 T cell and NK cells.

HIV infection perturbs the expression of activating and inhibitory receptors on NK cells resulting in the modulation of NK cell function. For example, NK cells from HIV infected patients have increased expression of receptors like CD94, CD161 and KIR2DL1 (90, 91), and decreased expression of NKp44 and 2B4 (92, 93). The molecular mechanisms that generate NK receptor expression modulation during HIV infection remain unknown. Possible causes include HCMV co-infection or chronic activation of immune system following loss of containment of gut commensal flora.

NK cell activation can be modulated by a 'cross-talk' between NK cells and other immune cells, especially dendritic cells. pDCs are the major type I IFN producing cells during viral infection, and IFN α/β primes NK cells for cytolytic activity. During the course of disease, HIV-infected pDC are progressively depleted resulting in a decrease of IFN- α levels (94, 95). This may be one mechanism behind the suppression of NK-mediated lysis of autologous infected CD4+ T cells in HIV infected patients. 'Classical' cDC from HIV patients produce less IL-12 and subsequently fail to activate NK cells for IFN- γ and TNF- α

secretion (96, 97). Saidi et al. demonstrated that the cytokine High Mobility group Box protein 1 (HMGB1) plays a role as an immune 'alarmin' during HIV infection (98). HMGB1 is an endogenous danger signal produced by innate effectors such as NK cells, which can be released during states of cellular stress or damage. HMGB1 normally promotes the NK-DC cross talk but, in the context of chronic HIV infection, triggers viral replication in DC and blocks NK-mediated killing of infected DC thus contributing to viral persistence (99). Increased levels of circulating HMGB1 are detected in HIV-1-infected individuals and are associated with high viral loads (100). Lastly, FoxP3⁺ Tregs can suppress both T and NK cell responses, and increased numbers of activated Tregs have been observed in HIV patients (101). Treg suppression of NK cell function may be mediated by TGF- β dependent NKG2D down-regulation (102). Moreover, circulating levels of other immunosuppressive cytokines (like IL-10) are increased in HIV infected patients (103). Collectively, the dysregulated NK cell 'cross-talk' results in reduced NK cell functions and an inefficient anti-viral response during chronic HIV infection.

While it is generally assumed that NK cells play a critical role in the control of HIV infection, in some cases, a paradoxical role for NK cells in promoting HIV replication or pathology has been observed. A conserved peptide in gp41 (called 3S) can induce the expression of a NKp44 ligand (NKp44L) on non-infected CD4⁺ T cells rendering them targets for NK cell elimination (104). Nef can inhibit NKp44L expression thereby protecting infected CD4⁺ T cells from NK cell lysis (105). Blocking NKp44L expression by anti-3S antibodies protects autologous CD4⁺ T cells in SHIV-infected macaques from NK cell cytotoxic activity, and can preserve CD4 central memory T cells (106). Viral proteins may therefore re-configure innate immune cell function to promote viral persistence.

14.3.3. Advances in HIV therapies that target NK cells

In the past three decades, tremendous efforts have been devoted to create an efficient vaccine to combat HIV. The discovery of new broadly neutralizing Abs and vectorbased vaccines to induce cell-mediated immunity provide hope, however we still lack an efficient vaccine that can induce HIV specific adaptive immunity. In recent years, the role for innate immune responses, and especially those mediated by NK cells, in the early resistance to HIV and in the control of persistent infection have been extensively studied. While HARRT treatment can provide an important degree of viremic control, NK cell numbers and functions are generally not normalized during treatment suggesting the existence of additional mechanistic deficiencies (103, 107-109).

Several new approaches have been proposed to restore NK cell function in HIVinfected individuals. Expression of inhibitory receptors on NK cells increases in HIV infected patients, and blocking these receptors with mAbs or small molecule compounds may help increase NK cell activation. Indeed, mAb masking of inhibitory KIRs increases the cytolytic activities against autologous infected cells (110). Still, NK cells may be generally activated by this approach, and potential side effects need to be carefully assessed before using this approach in HIV patients.

As cytokine levels are dysregulated in HIV infected patients, exogenous delivery of IL-2, IL-15, IL-21, and/or IFN- α/β may help boost the NK cell homeostasis and function. In particular, IL-2 has been approved clinically as a therapy to treat metastatic melanoma and renal cancers (111). Although the treatment is associated with dose dependent toxicity, anti-tumor NK and cytotoxic T cell responses are significantly improved. Still, immune cells are activated and Tregs are expanded during IL-2 treatment (112), so this treatment may lead to increased HIV infection and higher viral load. The combination of HAART and lowdose IL-2 treatment may minimize the viral production while enhancing the antiviral activities. IL-15 also holds promise as an adjuvant for HIV therapy. Compared to IL-2, IL-15 is less toxic and has lower mitogenic potential (113). IL-15 inhibits apoptosis of NK and CD8 T cells from HIV infected patients by up-regulating the expression of Bcl-X_L. However, recent animal study suggests that although transient stimulation of IL-15/IL-15Ra complexes in vivo induces NK cell proliferation and activation, prolonged stimulation may impair NK phenotype and function (114). As with IL-2, the dose and duration of IL-15 treatment must be carefully considered. IL-21 can strongly enhance ex vivo NK cells functions and prevents apoptosis with minimal stimulation of HIV replication (115). Lastly, as pDC numbers and functions are compromised in HIV infected patients, exogeneous IFNα/β priming of NK cell cytolytic activity could explain the viral suppression and decreased viral integration in treated HIV patients (116).

14.4. Human immune system (HIS) mice: a tool to study human NK cell biology in vivo

14.4.1 Human NK cell reconstitution in HIS mice

Given the central role of NK cells in maintaining immune homeostasis and for immune responses during infection, inflammation and cancer, an in vivo model that can allow a better understanding of human NK cell development and function has multiple applications. Human Immune System (HIS) mice are generated after transfer of human CD34⁺ cells into suitably conditioned immunodeficient mice. Several different HIS mouse models are available and the reader is referred to recent reviews that describe these models in detail (117). Most HIS mice demonstrate clear evidence of multi-lineage human
hematopoietic reconstitution, with strong development of lymphoid cells and lower levels of myeloid cell reconstitution. Still, within the lymphoid lineage, not all lymphocyte subsets are reconstituted with equal efficiency. Human B and T cells are in general, well represented in HIS mice. In contrast, human NK cell reconstitution, as well as other innate lymphocyte subsets (gd T cells, NK-T cells) remains relatively low in these models.

Despite the low levels of NK cell reconstitution, several studies managed to characterize the phenotypes and functions of the few NK cells that develop in NOD-based (NOG and NSG) HIS mice. Human NK cells are present in bone marrow and spleen of reconstituted NOG mice, and show cytolytic function against K562 tumor cells at high effector to target (E:T) ratios (118). Human NK cells from CD34⁺-transplanted NSG mice and supplemented with human IL-7 were generated that lack the surface expression of inhibitory receptors like KIRs. Most of these human NK cells displayed a CD56^{hi}CD16⁻ phenotype and appeared functional inert as they failed to kill K562 cells at low E:T ratios and only poorly produced IFN- γ after stimulation (119). Another study showed that almost half of NKp46⁺ NK cells in NSG mice are CD56- and require in vivo or in vitro activation to become CD56⁺ (120).

Similarly, systemic NK reconstitution in BALB/c Rag2^{-/-}Il2rg^{-/-} (BRG) recipients is poor in terms of absolute numbers of CD3⁻NKp46⁺ cells (38). In contrast with NSG and NOG mice, CD56⁺CD16⁺ NK cells can be found in BRG mice but within the thymus and lymph nodes (121, 122). Still, human NK cells from BRG mice express NKp46, NKG2D, CD94 and KIRs, similar to their native human counterparts. The normal expression of CD122, NKG2A and CD161 suggest a functional competence of human NK cells in BRG mice that is confirmed by the degranulation displayed when co-cultured with tumor cell lines (38, 123).

In the NOD–SCID–bone marrow–liver–thymus model (BLT mice), T cell development is prominent and highly efficient due to T cell education in a human thymic microenvironment. This results in a systemic T cell reconstitution (lymphoid and non-lymphoid tissue, including gut, salivary glands, skin) that generates strong adaptive T cell responses. Nevertheless, NK cell reconstitution in BLT mice is still poor in terms of number of CD3⁻CD56⁺ NK cells (124).

14.4.2. Approaches to improve human NK cell reconstitution and function in HIS mice

NK cells represent 10-15% of lymphocytes in human blood, whereas current HIS models rarely achieve levels beyond 1-3% CD56⁺ NK cells within the human CD45⁺ cell gate. Multiple mechanisms may explain the poor human NK cell reconstitution and function

in HIS mouse models. One possibility is that IL-15 availability differs in the different recipient strains. NOD mice have a defective IL-15 allele, which normally contributes to the NK cell defect in this strain and might also affect human NK cell development in NSG HIS mice (125). While the IL-15 allele in the BALB/c background is functional, the levels of human NK cells in BRG HIS mice are not dramatically improved compared to NSG HIS mice. This observation raises the intriguing possibility that mouse IL-15 might not be the driving force behind human NK cell development in HIS mice. While mouse IL-15 can cross-react weakly with human cells (38), human IL-15 is more efficient in stimulating IL-15-reponsive lymphocytes. Interestingly, in vivo neutralization of mouse IL-15 had little effect on human NK cell development (38). Moreover, exogenous treatment of HIS mice with human IL-15 was shown to strongly improve human NK cell development and function in HIS mice (38, 122, 126). These observations predict that approaches that increase IL-15 'transpresentation' will improve human NK cell homeostasis in HIS mice.

Another difference in HIS models involves the host macrophage response to xenografted cells. SIRPa is an inhibitory receptor expressed by macrophages that recognizes CD47 expressed on most nucleated normal cells (127). The interaction between SIRPa and CD47 delivers a 'Do not eat me' signal to the macrophage and thereby protects the CD47+ target cell from phagocytosis. Human CD47 poorly interacts with most mouse SIRPa alleles, although in NOD mice, SIRPa^{NOD} binds human CD47 with high affinity and provides a mechanism to explain the higher reconstitution of human xenografts in NODbased immunodeficient mice (127). As such, another possible reason for the poor human NK cell development in BRG HIS mice could be the enhanced phagocytosis of human NK cells due to poor SIRPa-CD47 interactions. This hypothesis has been tested using three different approaches: forced expression of mCD47 on human cells before reconstitution of BRG mice, creation of BRG SIRPa^{NOD} congenic mice and creation of BRG hSIRPa transgenic mice (128, 129). In all these cases, human NK cell numbers are increased significantly but the frequencies of these cells are still low compared to that observed in human peripheral blood. Clearly additional mechanisms play a role in this process and further work is required in this area.

A plethora of hematopoietic and non-hematopoeitic cells intimately regulate NK cell development and function. NK differentiation involves the interaction of NK cells with stromal cells and DC, pDC, Treg and myeloid cells (130-132). However, many of these cell types are not efficiently reconstituted in HIS mice and stromal cells are by definition of mouse origin in the HIS model. Studies have shown that distinct cytokines can selectively boost the engraftment of DC and Tregs in HIS mice (126, 133, 134). In the future, it will be interesting to systematically study the effects of diverse human cytokines (alone or in

combination) for NK cell homeostasis and function in HIS mouse; for these studies, hydrodynamic injection may offer a flexible approach to rapidly test cytokine effects.

14.4.3. Future development of HIS mice to study NK cell responses in HIV infection

HIV tropism is limited to human and chimpanzees (135). As many countries either ban or severely restrict the research on great apes, there is a pressing need to identify alternative in vivo models that can recapitulate the kinetics of HIV infection in man, and can be used to develop new therapeutics and screen vaccine candidates. A relatively simple, reproducible, small sized and less costly animal model, ideally based in rodents, could provide a means to better understand HIV pathophysiology and test new therapies.

In recent years, HIS mice have been used to model human HIV infections (135-138). HIS mice have several unique advantages for the study of HIV. First, multiple cohorts of HIS mice can be generated from multiple, genetically distinct donors. This overcomes the intragenetic variables generated from clinical studies on pooled data from patient samples but also allows for analysis of genetic differences. Second, the continuous supply of newly generated immune cells in vivo during the course of HIV infection enables longitudinal studies on HIV viral persistence and evolution. Third, both laboratory-adapted and clinical isolates of HIV virus can be used in HIS mice to study HIV transmission and human immune response. Fourth, the access to all immune tissues, and in particular mucosal sites, will allow in-depth investigation of the biology of HIV, which has been limited in humans because of the difficulty in obtaining biopsies from infected patients. Moreover, multiple routes of HIV transmission have been shown to elicit pathology in HIS mice. Finally, new therapeutic or prophylactic approaches may be tested in HIS mice because of the capacity of these models to recapitulate normal human immune responses.

In recent years, HIS mice have provided a better understanding of the early events of HIV infection and represent a useful platform to test new therapeutic approaches(139-142). The success in modeling HIV infection in HIS mice has been tempered by the relatively weak anti-HIV adaptive immune responses that can be elicited in this context (136-138). It is not known whether anti-HIV NK cell responses occur in infected HIS mice, partly due to the inadequate reconstitution of human NK cells in HIS mice in general. Some of the aforementioned strategies to boost NK cell homeostasis in HIS mice should facilitate the study of NK cells during HIV infection.

As alluded to earlier, it is not fully understood if NK cells are beneficial or detrimental in the control of HIV infection. HIS mice offer a means to dissect the role for NK cells in HIV biology. Whether human NK cells eliminate virally-infected T cells and DC or alternatively whether they attack uninfected human CD4 T cells expressing NKp44 ligands induced by

gp41-derived peptides can be tested. Once established, blocking antibodies or compounds could be tested to modulate the beneficial of detrimental effect from human NK cells on the evolution of HIV infection and disease.

Harnessing the power of NK mediated ADCC in HIS model may be a new approach to identify novel HIV therapeutics. Previous studies on antibody responses against HIV infection were focused on neutralizing effect to block the entry of HIV viruses. Recent findings indicate that many previously identified non-neutralizing antibodies may actually play a role via ADCC. HIS mice with boosted NK cells could offer an in vivo model to evaluate the ADCC ability of those antibodies. In addition, as activated and proliferating T cells are the major targets for HIV infection, administration of anti-CD25 antibody may specifically deplete those cells by NK mediated ADCC. This hypothesis can be tested using HIS mice boosted with human NK cells.

The absence of appropriate HLA class I molecules in most HIS models poses an issue for proper human NK cell education and function. In current HIS models, NK cells express low levels of KIRs, produce less IFN-γ and display lower cytolytic activity after stimulating with cytokines and tumor cells. In addition to the lack of appropriate cytokines, the absence of correct 'educating' HLA-KIR interactions may also account for these phenotypic and functional NK cell deficiencies observed in HIS mice. The use of HLA class I expressing HIS mice (143, 144) may provide a means to study the impact of MHC-KIR interactions to shape NK mediated responses during HIV infection.

Vaginal and rectal transmission of HIV accounts for the majority of new transmissions and early virus replication occurs primarily in gastrointestinal tract (135). As such, NK mediated innate immunity may be critical for the early control of HIV replication at mucosal sites. However, the reconstitution of human NK cells at these sites is rather poor in most HIS models. Whether this is secondary to poor lymphoid structure development (145), or to defective NK cell maturation and homing, remains unclear. Alternatively, activation of the murine innate system during HIV infection, due to indirect effects of human cytokines that cross-react with mouse hematopoietic and stromal components, may also dampen human innate (and adaptive) immune responses. These are challenging issues for the future use and development of HIS mice to study HIV infection and disease pathogenesis.

14.5. Concluding remarks

Humanized immune system (HIS) mouse models provide a novel tool to better understand the contribution of NK cells to the immune response against HIV. Functional subsets of human NK cells can develop in HIS mice; these same mice have been shown to be permissive for HIV infection, replication and its subsequent disease manifestations. HIS mice can therefore provide a basis to experimentally decipher the role for human NK cells in HIV pathophysiology. The ability to modulate NK cell function in HIS mice constitutes a tractable translational tool that could contribute to the development NK cell-based therapies and vaccines that limit HIV replication or accelerate the immune control of the disease.

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Cell Reports

NFIL3 Orchestrates the Emergence of Common Helper Innate Lymphoid Cell Precursors

Graphical Abstract



Highlights

- Cell-intrinsic Nfil3 ablation results in impaired development of ILC subsets
- NFIL3 deficiency leads to loss of common helper-like ILC progenitors (CHILPs)
- NFIL3 is controlled by mesenchyme-derived IL-7 in lymphoid precursors
- NFIL3 exerts its function in CHILP via direct regulation of Id2

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In Brief

Innate lymphoid cells (ILCs) originate from a common innate lymphoid cell progenitor. However, the transcriptional program that sets the identity of the ILC lineage remains elusive. Xu et al. show that NFIL3 acts downstream of IL-7 signaling, regulating the emergence of common helper ILC progenitors via direct regulation of Id2.



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NFIL3 Orchestrates the Emergence of Common Helper Innate Lymphoid Cell Precursors

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SUMMARY

Innate lymphoid cells (ILCs) are a family of effectors that originate from a common innate lymphoid cell progenitor. However, the transcriptional program that sets the identity of the ILC lineage remains elusive. Here, we show that NFIL3 is a critical regulator of the common helper-like innate lymphoid cell progenitor (CHILP). Cell-intrinsic Nfil3 ablation led to variably impaired development of fetal and adult ILC subsets. Conditional gene targeting demonstrated that NFIL3 exerted its function prior to ILC subset commitment. Accordingly, NFIL3 ablation resulted in loss of ID2⁺ CHILP and PLZF⁺ ILC progenitors. Nfil3 expression in lymphoid progenitors was under the control of the mesenchyme-derived hematopoietin IL-7, and NFIL3 exerted its function via direct Id2 regulation in the CHILP. Moreover, ectopic Id2 expression in Nfil3-null precursors rescued defective ILC lineage development in vivo. Our data establish NFIL3 as a key regulator of common helper-like ILC progenitors as they emerge during early lymphopoiesis.

INTRODUCTION

The immune system is composed by myriads of cell types and lymphoid organs that ensure immune surveillance and protective immunity. The adaptive immune system arose late in evolution and consists of B and T lymphocytes that express recombining antigen-specific receptors. Naive T and B cells are activated by their cognate antigen in secondary lymphoid organs and undergo significant cell division and differentiation before exerting their effector function. In contrast, innate lymphocytes display rapid effector functions despite their set of limited germ-line-encoded receptors. For more than three decades, natural killer (NK) cells were the only recognized innate lymphocytes (Diefenbach et al., 2014; McKenzie et al., 2014; Spits et al., 2013). More recently, additional innate lymphocytes have been discovered and were considered to be part of a family of effector cells collectively named innate lymphoid cells (ILCs) (Diefenbach et al., 2014; McKenzie et al., 2014; Spits et al., 2013).

ILCs have a lymphoid morphology, lack rearranged antigen receptors, and are abundantly present at mucosal surfaces, such as the enteric lamina propria. The expression of lineagespecific transcription factors and discrete cytokine profiles led to the identification of three distinct ILC subsets that have striking parallels with T helper (Th) cell subsets. Group 1 ILCs (ILC1) resemble Th1 cells and include NK cells and other IFN_Y-producing innate effectors ILC1 (Bernink et al., 2013; Diefenbach et al., 2014; Fuchs et al., 2013; Klose et al., 2014; McKenzie et al., 2014; Spits et al., 2013; Vonarbourg et al., 2010). ILC1s were shown to depend on TBX21 (T-bet), IL-7, and IL-15 (Diefenbach et al., 2014; McKenzie et al., 2014; Spits et al., 2013). Group 2 ILCs are similar to Th2 cells. ILC2s are $ROR\alpha$ (Halim et al., 2012; Wong et al., 2012) and GATA3 (Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjösberg et al., 2012) dependent, IL-7 dependent, and produce IL-5 and IL-13 (Moro et al., 2010; Neill et al., 2010; Spits et al., 2013). ILC2s have been shown to play important roles in helminth infections, asthma, and allergy contexts (McKenzie et al., 2014; Spits et al., 2013). Group 3 ILCs (ILC3) are RORyt and partly AhR dependent, rely on IL-7, and similarly to Th17 cells produce IL-17 and IL-22 (Diefenbach et al., 2014; Kiss et al., 2011; Lee et al., 2012; McKenzie et al., 2014; Qiu et al., 2012; Spits et al., 2013). ILC3s were also shown to mediate inflammatory







Figure 1. Nfil3 Deficiency Results in Reduced Adult and Fetal ILCs

(A) Fetal liver (FL) CLP, BM CLP and NK, BM and liver ILC1, BM and lung ILC2, gut ILC3, fetal gut (FG) CD4⁻ LTi, FG CD4⁺ LTi, and B and T cells were analyzed by

(c) relative (C) CC⁺, bit of the and the one of the

(E) Number of BM, lung, and gut ILC2s from Nfi/3^{+/+} and Nfi/3^{-/-} mice. BM: Nfi/3^{+/+} n = 6; Nfi/3^{-/-} n = 6; Lung: Nfi/3^{+/+} n = 8; Nfi/3^{-/-} n = 8; gut: Nfi/3^{+/+} n = 3; Nfi/3^{-/-} n = 3.

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bowel diseases (Buonocore et al., 2010; Vonarbourg et al., 2010) and to control immune responses to attaching and effacing enteric pathogens such as *Escherichia coli* and *Citrobacter rodentium* (Sonnenberg et al., 2011; Zheng et al., 2008). Lymphoid tissue inducer (LTi) cells are the prototypical member of ILC3s, are dependent on retinoic acid signaling, and were shown to play a critical role in secondary lymphoid organ (SLO) development and tissue homeostasis (Diefenbach et al., 2014; Spencer et al., 2014; Spits et al., 2013; van de Pavert et al., 2014).

ILCs express the transcriptional regulator inhibitor of DNA binding 2 (ID2), and Id2 deficiency leads to developmental block of ILCs (Boos et al., 2007; Moro et al., 2010). ID2 is a helix-loophelix factor that was shown to sequester E proteins from their target gene promoters. Interestingly, EBF1 has been shown to counter Id2 expression (Thal et al., 2009), and conditional deletion of *Ebf1* in committed pro-B cells leads to their conversion into different ILC subsets (Nechanitzky et al., 2013), suggesting the existence of a common ILC precursor. Interestingly, a $\alpha 4\beta 7^{+}\text{PLZF}^{+}$ cell was recently identified as a committed precursor to ILCs with the exception of NK and LTi cells (Constantinides et al., 2014) and another study demonstrated that a $\alpha 4\beta 7^{\text{+}}\text{ID2}^{\text{high}}$ cell was the common helper-like innate lymphoid precursor (CHILP) to all helper-like ILCs (Klose et al., 2014). Nevertheless, the factors that control the emergence of these recently described ILC precursors from common lymphoid progenitors (CLPs) remain unknown. Altogether, these data suggest the existence of additional, yet unrecognized, transcriptional regulators that set the identity of the CHILP (Klose et al., 2014).

NFIL3 (also known as E4BP4) is a basic leucine zipper transcription factor that was identified by its DNA-binding activity (Zhang et al., 1995). NFIL3 coordinates signals from several regulatory pathways, including the circadian clock. More recently. NFIL3 was shown to mediate several immune processes. NFIL3 controls pro-B cell survival (Ikushima et al., 1997), IgE class-switch (Kashiwada et al., 2010), Th2 and Th17 cytokine expression (Kashiwada et al., 2011a; Motomura et al., 2011; Yu et al., 2013), IL-12 regulation (Kobayashi et al., 2011; Smith et al., 2011), and CD8a dendritic cell development (Kashiwada et al., 2011b). Interestingly, Nfil3-null mice have an early block in NK cell development that perturbs ID2, GATA3, EOMES, and TBX21 expression in hematopoietic precursors (Gascoyne et al., 2009; Kamizono et al., 2009; Male et al., 2014); these different transcription factors critically control NK cell differentiation and homeostasis (Spits et al., 2013).

In this report, we show that cytokine-dependent expression of NFIL3 promotes the development of the CHILP via direct regulation of *Id2*. Based on our results, NFIL3 emerges as a key transcription factor that orchestrates the emergence of ILC precursors from CLPs.

RESULTS

Nfil3 Is Expressed by All Helper-like ILCs and Is Required for ILC Homeostasis

NFIL3 is an essential transcription factor for NK cell commitment from lymphoid progenitors. *Nfil3*-deficient mice have a profound defect in peripheral NK cell homeostasis, which arises from an early block in NK cell maturation in the bone marrow (Crotta et al., 2014; Gascoyne et al., 2009; Male et al., 2014; Seillet et al., 2014a). As diverse ILCs are thought to arise from lymphoid precursors via common intermediates (Constantinides et al., 2014; Klose et al., 2014; Spits et al., 2013), we hypothesized that *Nfil3* might have generalized roles in ILC development.

We first assessed *Nfil3* expression in lymphoid precursors and distinct ILC subsets from fetal and adult tissues. We found high levels of *Nfil3* transcripts in all ILC subsets, whereas adaptive B and T lymphocytes expressed very low levels of *Nfil3* (Figure 1A). In agreement, *Nfil3*^{GFP} reporter mice revealed that GFP was clearly expressed by NK cells and their immature BM precursors, whereas B and T cells were GFP^{Io} (Figures 1B and S1A). Interestingly, helper ILC1, ILC2, and ILC3 subsets from diverse fetal and adult tissue sites also expressed high levels of GFP (Figures 1B and S1A), a finding also confirmed at the protein level using intracellular staining for NFIL3 in adult NK, ILC1, ILC2, and ILC3 subsets (Figure S1B). These data demonstrate that NK cells are not the only lymphoid cell subset that strongly expresses *Nfil3* but that high constitutive *Nfil3* expression is a common characteristic of all ILC subsets.

To assess the role of NFIL3 on the development and homeostasis of helper-like ILC1. ILC2, and ILC3, we analyzed mice with a germ-line deletion of Nfil3 (Gascoyne et al., 2009). In the absence of Nfil3, ILC1 in the BM and gut, ILC2 in the BM and lung, and CD4⁺ ILC3 in the gut were all clearly reduced, whereas CLPs were not affected (Figures 1C-1F). Germ-line deletion of Nfil3 also resulted in a decrease of ROR_Yt⁺ ILC3 subsets in the fetal liver, gut, and lymph nodes with a clear dose-dependent effect (Figures 1G and S1C-S1F). Consequently, Nfil3 ablation resulted in reduced number of minute PPs and severely diminished fetal LN size (Figures S1G-S1I). Of note, PPs were also reduced in Nfil3^{-/-} adult animals, arguing against a putative PP developmental delay (Figure S1G). Taken together, these data confirm recent reports on the broad role for Nfil3 in controlling the homeostasis of helper ILC subsets and NK cells in both fetal and adult life (Geiger et al., 2014; Seillet et al., 2014b; Yu et al., 2014).

Hematopoietic-Autonomous NFIL3 Controls ILC Development before Commitment into Discrete ILC Lineages

Nfil3 is widely expressed by tissues from different germ layers having significant pleiotropic effects. In order to determine whether hematopoietic cell-intrinsic *Nfil3* expression is required for ILC homeostasis, we ablated *Nfil3* in a lineage-specific

⁽F) Number of gut NCR⁺ (NKp46⁺), NCR⁻ (NKp46⁻CD4⁻), and CD4⁺ ILC3 populations from *Nfil3*^{+/+} and *Nfil3*^{+/-} mice. *Nfil3*^{+/+} n = 6; *Nfil3*^{-/-} n = 7. (G) Left: number of $\alpha 4\beta 7^{high}$ ROR γt^{+} FL cells. *Nfil3*^{+/+} n = 5; *Nfil3*^{+/-} n = 9; *Nfil3*^{-/-} n = 8. Right: percentage of FG CD4⁻ and CD4⁺ LTi cells. *Nfil3*^{+/+} n = 5; *Nfil3*^{+/-} n = 9; *Nfil3*^{-/-} n = 8.

Error bars show SE.*, **, and *** p values for Student's t test lower than 0.05, 0.01, and 0.001, respectively. See also Figure S1.



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fashion. *Nfil3^{fl/fl}* mice were bred to *Vav1*-iCre mice, ensuring Cre activity in the hematopoietic lineage (de Boer et al., 2003; Motomura et al., 2011; Figure 2A). Analysis of adult *Vav1-i*Cre.*Nfil3^d* mice revealed unperturbed BM CLP development, whereas helper ILC1, ILC2, and ILC3 subsets were reduced when compared to their *Nfil3^{fl/fl}* littermate controls (Figures 2B and 2C). Consistent with observations in germ-line *Nfil3^{-/-}* mice, fetal gut ILC3 subsets were significantly reduced in E15.5 Vav1-iCre.*Nfil3^{d/d}* embryos when compared to their *Nfil3^{fl/fl}* littermate controls (Figures 2D, S2A, and S2B).

Having established the important function of NFIL3 in overall ILC homeostasis, we next assessed whether NFIL3 is still required upon commitment into mature ILC subsets. In order to test this hypothesis, we analyzed mice in which *Nfil3* was ablated after commitment into the ILC3 lineage using *Rorc*-Cre mice (Eberl and Littman, 2004; Figure 2A). Strikingly, analysis of adult and fetal *Rorc*-Cre.*Nfil3*^{4/4} mice demonstrated normal enteric ILC3 development (Figures 2E, 2F, and S2B), indicating that NFIL3 exerts its hematopoietic cell-intrinsic function before *Rorc* acquisition but appears dispensable once ILC3s become lineage committed.

Further evidence that NFIL3 is required before ROR γ t acquisition was provided by in vitro differentiation assays. The fetal gut harbors ILC precursors Lin⁻IL7Ra⁺a4 β 7⁺ID2⁺ROR γ t⁻ and CD4⁻*Rorc*-GFP⁺ LTi that can further differentiate to CD4⁺ LTi cells when co-cultured with OP9 cells (van de Pavert et al., 2014). Whereas these ILC precursors from *Nfi/3^{+/-}* failed to give rise to committed ILC3s (*Rorc*-GFP⁺), *Nfi/3^{+/-}* CD4⁻ LTi (*Rorc*-GFP⁺) could efficiently differentiate into CD4⁺ LTi cells under the same conditions (Figures 2G and 2H). Thus, hematopoiet-ic-autonomous NFIL3 expression in uncommitted fetal ILC precursors is critical for their further maturation.

NFIL3 Regulates the Emergence of Common Helper-ILC Precursors

Given the broad impact of NFIL3 on ILC homeostasis and the evidence that NFIL3 exerted its role prior to ILC lineage commitment, we hypothesize that NFIL3 may be required during the generation of committed ILC precursors (Constantinides et al., 2014; Klose et al., 2014). Common-helper-like ILC precursors (CHILPs) have been defined as Lin⁻IL-7Ra⁺α4β7^{high}ID2⁺ cells that express variable amounts of PLZF (Constantinides et al., 2014; Klose et al., 2014). Using *Id2* reporter mice (Rawlins

et al., 2009), we found that *Nfi/3* has a critical, dose-dependent role in the development of fetal and adult ID2⁺ CHILPs (Figures 3A and 3B). Similarly, $\alpha 4\beta 7^{high}$ PLZF⁺ progenitors were severely reduced in the BM and fetal liver of *Nfi/3* germ-line-deficient and *Vav1-i*Cre.*Nfi/3^{Δ/Δ}* mice (Figures 3C, 3D, S3A, and S3B). In line with these findings, $\alpha 4\beta 7^+$ ILC precursors expressed higher levels of *Nfi/3* transcripts, *Nfi/3*^{GFP}, and NFIL3 protein when compared to CLPs (Figures 3E and 3F).

The observation that CHILPs were strongly reduced in the absence of Nfil3 provides an explanation for the broad effects of Nfil3 in ILC homeostasis. Nevertheless, despite the apparent lack of CHILPs in Nfil3-deficient mouse models, some peripheral ILC2s and ILC3s were still present in the gut and lung (Figure 1). This finding could suggest a CHILP-independent pathway of ILC2 and ILC3 development. Alternatively, despite strongly reduced CHILPs in Nfil3-/- mice, these rare CHILPs further develop into ILC2s and/or ILC3s and expand in the periphery. In order to address these possibilities, we performed competitive BM reconstitution experiments using lethally irradiated hosts (CD45.1), which received WT (CD45.2) or Nfil3^{-/-} (CD45.2) BM against a WT competitor (CD45.1/2) in a 1:1 ratio (Figure 4A). Analysis of such chimeras 8 weeks after transplantation revealed that, despite normal CLP development, $\alpha 4\beta 7^{high}~PLZF^-$ and PLZF⁺ CHILPs derived from Nfil3^{-/-} precursors were significantly reduced when compared to their WT counterparts (Figures 4B and S4). In line with the normal CLP development in the absence of NFIL3, thymic T cell development from Nfil3-/ precursors was indistinguishable from WT (Figure 4C). In contrast, all mature ILC subsets (ILC1, ILC2, and ILC3) that derived from $\textit{Nfil3}^{-/-}$ precursors were consistently and severely reduced in these chimeras (Figure 4D). Altogether, these data further confirm that Nfil3 acts in a hematopoietic cell-intrinsic fashion to drive ILC development. In addition, these results provide compelling evidence that NFIL3 is a critical regulator of ILC progenitors in early lymphopoiesis as they emerge from CLPs.

Nfil3 Expression Is Modulated by the $\gamma_{\text{c}}\text{-Dependent}$ Cytokine IL7

Early studies of NFIL3 implicated its role in regulating the survival of lymphoid cells in response to IL-3 (lkushima et al., 1997). Therefore, we interrogated whether NFIL3 acts downstream of critical cytokines required for early stages of ILC development. Lymphoid precursors were isolated from *Nfil3*^{GFP} mice and

Figure 2. NFIL3 Acts in a Cell-Autonomous Fashion before Commitment into Mature ILCs

(A) Conditional Nfil3-deficient animals breeding scheme.

Error bars show SE. *, **, and *** p values for Student's t test lower than 0.05, 0.01, and 0.001, respectively. See also Figure S2.

⁽B and C) Conditional *Nfil3*-deficient animals were bred with *Vav1*-iCre animals. Flow cytometry analysis of liver NK and ILC1; BM CLP and ILC2; enteric ILC2 and total ILC3; and NCR⁺, NCR⁻, CD4⁺ ILC3 sub-populations from adult conditional *Nfil3*-deficient animals and their littermate controls. Frequency of NK, ILC1, and BM ILC2 from Lin⁻ cells: NK *Nfil3^{-/-}* = 2.9%, ILC1 *Nfil3^{+/+}* = 2.5%, *Nfil3^{-/-}* = 0.3%; ILC2 *Nfil3^{+/+}* = 0.5%, *Nfil3^{-/-}* = 0.03%. Frequency of NCR⁺, NCR⁻, and CD4⁺ ILC3 from Lin⁻ THY1⁺ cells: NCR⁺ ILC3 *Nfil3^{+/+}* = 5.3%, *Nfil3^{-/-}* = 0.2%; NCR⁻ ILC3 *Nfil3^{+/+}* = 3.19%, *Nfil3^{-/-}* = 5.6%; CD4⁺ ILC3 *Nfil3^{+/+}* = 5.6%, *Nfil3^{-/-}* = 2.9%.

⁽D) Flow cytometry analysis and percentage of CD4⁻ and CD4⁺ LTi cells in E15.5 FG. $Nfil3^{n/n}$ n = 8; $Nfil3^{n/d}$ n = 7; $Nfil3^{d/d}$ n = 5.

⁽E) Conditional Nfi/3-deficient animals were bred with Rorc-Cre animals. Flow cytometry analysis and number of ILC3 in gut. Nfi/3^{///1} n = 3; Nfi/3^{///1} n = 3.

⁽F) Conditional *Nfil3*-deficient animals were bred with *Rorc*-Cre animals. Flow cytometry analysis and percentage of FG CD4⁻ and CD4⁺ LTi cells. *Nfil3*^{*dl/ll*} n = 7; *Nfil3*^{*dl/ll*} n = 5.

⁽G and H) $Nfil3^{+/-}$ mice were bred to $Rorc^{GFP}$ animals. E15.5 CD4⁻LTi cells and ROR_Yt⁻ ILC precursors from $Nfil3^{+/+}$ and $Nfil3^{+/-}$ were co-cultured with OP9. Flow cytometry analysis and percentage of ILC3 ROR_Yt⁺ (G) and CD4⁺ LTi (H) after 6 days of culture. $Nfil3^{+/+}$ n = 4; $Nfil3^{+/-}$ n = 8.



Figure 3. NFIL3 Is Required for the Emergence of CHILP and $\alpha 4\beta 7^{high} PLZF^+$ ILC Progenitors

(A and B) Flow cytometry analysis and number of CHILP cells in BM and FL from $Nfil3^{+/+}$ and $Nfil3^{-/-}$ mice. BM: $Nfil3^{+/+}$ n = 4 and $Nfil3^{-/-}$ n = 5; FL: $Nfil3^{+/+}$ n = 5 and $Nfil3^{-/-}$ n = 6.

(C) Number of $\alpha 4\beta 7^{high}$ PLZF⁺ ILC progenitors in BM from *Nfil3*^{+/4} and *Nfil3*^{-/-} mice and conditional deficient animals and their littermate controls. BM: *Nfil3*^{+/4} n = 6; *Nfil3*^{-/-} n = 3; BM: *Nfil3*^{4/4} n = 3; *Nfil3*^{4/4} n = 3.

(D) Number of FL CHILP PLZF⁺ progenitors in E15.5 Nfi/3^{+/+} and Nfi/3^{-/-} embryos. FL: Nfi/3^{+/+} n = 5; Nfi/3^{+/-} n = 5.

(E) CLP and FLT3^{$-\alpha$ 4 β 7^{high} cells were analyzed by quantitative RT-PCR for *Nfil3* expression.}

(F) Flow cytometry analysis of Nfil3^{GFP} expression and NFIL3 in CLPs and FLT3^{- α 4 β 7^{high} from BM and E15.5 FL cells.}

Error bars show SE. * and *** p values for Student's t test lower than 0.05 and 0.001, respectively. See also Figure S3.

size of the resultant ILC2 colonies from $Nfi/3^{-/-}$ CLPs were significantly smaller compared to that obtained from WT CLP, which could not be explained by increased apoptosis rates or defective proliferation capacity (Figures 5E, 5F, and S5A–S5C). Noteworthy, this finding was also in agreement with normal proliferation and cytokine production of $Nfi/3^{-/-}$ ILC2 (Figures S5D and S5E). Interestingly, T cell colony sizes from $Nfi/3^{+/+}$ and $Nfi/3^{-/-}$ CLPs were similar (Figure 5E), suggesting an ILC-specific target for NFIL3 action. Taken together, these data suggest a model whereby the

stimulated with IL-7. We found that GFP expression was strongly induced by this cytokine in BM FLT3⁻ α 4 β 7^{high} ILC precursors as well as in BM CLP (Figure 5A). In line with these findings, lung ILC2 and gut NKp46⁺ ILC3 also upregulated *Nfi/3*^{GFP} expression, whereas mature NK cells and intestinal ILC1 did not modulate *Nfi/3*^{GFP} expression (Figures 5B and 5C). Binding of IL-7 to its cognate receptor triggers a signaling cascade, resulting in the activation of the JAK/STAT and PI3K/Akt pathways (Demoulin and Renauld, 1998). Thus, we asked whether IL-7 regulates *Nfi/3* in a STAT5-dependent fashion. We found that GFP levels were reduced in ILC that were cultured with STAT5 inhibitor, indicating that NFIL3 functions downstream of IL-7 and that the activation of STAT5 is required for IL-7-induced *Nfi/3*^{GFP} expression (Figure 5D).

As IL-7 could enhance NFIL3 expression in early lymphoid cell precursors and especially $\alpha 4\beta 7^{high}$ ILC precursors (Figure 5A), we hypothesized that cytokine-driven NFIL3 expression might regulate these cells as they emerge during early lymphopoiesis. Thus, we examined ILC2 generation in vitro from single *Nfil*3^{+/+} and *Nfil*3^{-/-} BM CLP. We observed that the average clone

 γ_c cytokine IL-7 regulates Nfil3 expression in common lymphoid precursors that impacts on these developing progenitors.

NFIL3 Directly Regulates Id2 in the CHILP

Because Id2+ ILC precursors were strongly reduced in Nfil3-deficient mice (Figure 3), we hypothesized that Id2 was a relevant downstream target of Nfil3 in CHILP. Strikingly, Id2 transcripts were strongly decreased in both fetal and adult Nfil3-/ ILC precursors and NFIL3 impacted on Id2 levels in FLT3-α4β7^{high} precursors, whereas Id2 levels were only moderately modulated in CLPs and mature ILC subsets (Figures 6A, 6B, and S6A). To gain additional insight into the regulatory mechanisms of Nfil3, we performed chromatin immunoprecipitation with a specific anti-NFIL3 antibody followed by quantitative PCR analysis (ChIP) in biologically relevant ILC progenitors ex vivo. NFIL3 bound the Id2 locus in CHILP, which also displayed active dimethylated H3K4 in the NFIL3-binding region (Figure 6C). Interestingly, whereas enrichment of NFIL3 binding was found at a region "D" close to the Id2 promoter in CHILP, NFIL3 bound to the distinct region "H" in more mature ILC2 and ILC3 (Figures



6C, S6B, and S6C), which also displayed active dimethylated H3K4 upstream of the NFIL3-binding region (Figure 6C). Noteworthy, NFIL3 did not bind to important genes to ILC2 and IL3 development, notably *Ahr*, *Tox*, *Notch2*, and *Rora* (Figure S6D). Despite this specific NFIL3 binding to the *Id2* locus in mature ILC subsets, *Nfil3* ablation did not affect *Id2* expression in these cells (Figure S6A), in agreement with the model that *Nfil3* expression is critical prior to, but not after, ILC commitment.

To further test whether NFIL3 controls CHILP generation via *Id2*, we determined whether differentiation of *Nfi/3*-deficient ILC in vivo could be restored by enforced *Id2* expression. We transduced *Nfi/3*^{+/+} and *Nfi/3*^{-/-} fetal liver progenitors with retroviral particles expressing *Id2* (pMig-*Id2*) or GFP only (empty vector) and generated bone marrow chimeras (Figure 6D). Retroviral transduction of *Id2* (pMig-*Id2*) allowed *Nfi/3*^{-/-} progenitors to reacquire their potential to differentiate into ILC1, ILC2, and ILC3 in vivo, whereas ectopic expression of *Id2* in WT progenitors had no impact in ILC lineages (Figure 6D). In agreement, retroviral transduction of *Id2* (pMig-*Id2*) allowed *Nfi/3*^{-/-} CLP to develop in vitro into PLZF⁺ CHILP when compared to their counterparts transduced with retroviral particles containing GFP only (Figure S6E). Collectively, these experiments demonstrate that NFIL3 directly regulates *Id2* expression in ILC progenitors and orchestrates their emergence from CLPs (Figure 6E).

DISCUSSION

The development of multiple and distinct hematopoietic cell lineages relies on tightly controlled expression of transcription

Figure 4. NFIL3 Regulates All Helper-like ILCs In Vivo through Regulation of the CHILP

(A) Competitive transplantation assays scheme. BM of Nfi/3^{+/+} and Nfi/3^{-/-} littermate controls were co-transplanted with equal numbers of third-party WT progenitors.

(B–D) Percentage of donor BM CLP, PLZF⁻, and PLZF⁺ progenitors and BM ILC2s (B); thymic DP, CD4, and CD8 cells (C); and BM and liver NK cells, BM and liver ILC1s, lung and gut ILC2s, and gut and spleen ILC3s (D). $Nfi/3^{+/+}$ n = 8; $Nfi/3^{-/-}$ n = 9. Error bars show SE. ** and *** p values for Student's t test lower than 0.01 and 0.001, respectively. See also Figure S4.

factors that promote lineage specification and commitment while suppressing alternative cell fates. As an example, several regulators induce the development of uncommitted hematopoietic progenitors into the B or T cell lineage. In contrast to the transcription factors that promote generation of adaptive lymphocytes, the factors that control ILC development are less well understood.

Diverse ILC subsets can be generated from CLPs, and ID2 has emerged as a central regulator of ILC fate (Hoyler

et al., 2012; Moro et al., 2010; Yokota et al., 1999). More recently, committed precursors to all helper ILCs (CHILPs) were identified within the fetal and adult Lin⁻IL-7Ra⁺a4 β 7^{high} progenitor cell population (Klose et al., 2014). Committed ILC precursors strongly express ID2 and harbor both PLZF⁺ and PLZF⁻ fractions (Klose et al., 2014), and NOTCH triggering could induce PLZF expression on PLZF⁻a4 β 7^{high} cells, suggesting a precursor-product relation between these subsets (Constantinides et al., 2014). PLZF-expressing ILC progenitors could give rise to ILC1, ILC2, and ILC3, but not LTi cells (Constantinides et al., 2014), whereas Id2⁺a4 β 7^{high} ILC precursors originate all help-er-ILC subsets, suggesting that α 4 β 7^{high}ID2⁺PLZF⁻ cells may harbor LTi potential. Nevertheless, how the emergence of CHILP from CLP is regulated remains elusive.

Whereas the transcriptional repressor ID2 is essential for development of all known ILC subsets, it is not clear how *Id2* expression is regulated in lymphoid progenitors (CLP) or how titration and reduction of E-protein activity allows for emergence of CHILP from these cells. Previous studies demonstrated that the transcription factor NFIL3 is broad regulator of ILC homeostasis (Gascoyne et al., 2009; Geiger et al., 2014; Male et al., 2014; Seillet et al., 2014b), although the molecular basis for the NFIL3 effect remained unclear. In this report, we demonstrate that NFIL3 is a critical regulator of the common-helper-like ILC progenitor (CHILP), while being dispensable for overall helper-like ILC fate and maintenance of discrete mature ILC subsets (Geiger et al., 2014; Seillet et al., 2014; Seillet et al., 2014b). We demonstrate that NFIL3 operated via direct regulation of *Id2* expression in ILC



precursors. In agreement, ectopic Id2 expression in vivo rescued developmental defects of ILC1, ILC2, and ILC3 from Nfil3-null lymphoid precursors. We further demonstrated that Id2 expression could rescue PLZF expression in Nfil3-/- CHILP, suggesting a transcription factor cascade that links Nfil3, Id2, and Zbtb16. Based on our results, NFIL3 emerges as a central regulator of the common helper ILC precursor in early lymphopoiesis. Interestingly, it was recently shown that NFIL3 could act in CLP upstream of Tox, also directing the development of a CXCR6+ common cytotoxic and helper ILC precursor (aLP: Yu et al., 2014). The relationship between αLP and CHILP is unclear, but it is possible that NFIL3 may act by distinct mechanisms in different ILC precursors. This notion is also in line with our own findings showing that NFIL3 binding occurred at different Id2 genomic regions in the CHILP and mature ILC3 and the absence of NFIL3 binding to Tox in ILC3 (Figures 6C and S6D).

A potent cell-intrinsic role for NFIL3 in the generation of all recognized ILC subsets, including NK cells (Gascoyne et al., 2009; Kamizono et al., 2009) and ILC1, ILC2, and ILC3 (this study; Geiger et al., 2014; Seillet et al., 2014b) has recently been reported. Our observation that NFIL3 is required for the generation of helper-ILC precursors, namely $\alpha4\beta7^{high}PLZF^-$ and $\alpha4\beta7^{high}PLZF^+$ precursors (Constantinides et al., 2014;

Figure 5. Nfil3 Expression Is Modulated by the $\gamma_{c}\text{-}\text{Dependent}$ Cytokine IL-7

(A–C) BM CLP and FLT3⁻ α 4 β 7^{high} (A), BM ILC2 and gut ILC3 (B), and spleen NK and liver ILC1 cells (C) from *Nfil3*^{GFP} mice were stimulated in vitro with IL-7 and analyzed by flow cytometry. (D) BM ILC2s from *Nfil3*^{GFP} mice were stimulated

(D) BM ILC2s from *Nfil3*^{GFP} mice were stimulated with IL-7 or IL-7 and STAT5 inhibitors and analyzed by flow cytometry.

(E) Flow cytometry analysis of ILC2 differentiated in vitro from *Nfil3^{+/+}* and *Nfil3^{-/-}* BM CLPs. iCD3, intracellular CD3.

(F) ILC2 and T cell number per colony. ILC2: $Nfi/3^{+/+} n = 37$; $Nfi/3^{-/-} n = 46$; T cells: $Nfi/3^{+/+} n = 49$; $Nfi/3^{-/-} n = 49$.

Error bars show SE. **p values for Student's t test lower than 0.01. See also Figure S5.

Klose et al., 2014), confirms a cellular mechanism for the broad effect of NFIL3 on multiple ILC subsets (Geiger et al., 2014). On a molecular level, we found that NFIL3 directly regulated Id2 in CHILP. ID2 is a transcriptional repressor that is critically required for NK cell and ILC development from hematopoietic precursors (Boos et al., 2007; Moro et al., 2010; Satoh-Takayama et al., 2010). We found that Id2 expression in Nfil3-/- CHILP was severely compromised and that NFIL3 bound specifically to the Id2 promoter in CHILP-remodeling chromatin configuration as revealed by specific enrichment of dimethylated H3K4. Moreover, ID2 appeared upstream

of Zbtb16 expression in ILC precursors, allowing us to propose a NFIL3 > ID2 > PLZF transcription factor cascade that regulates ILC emergence from CLPs.

Our data indicate that cellular expansion of *Nfil3^{-/-}* ILC precursors toward ILC2 was less robust, although it could not be explained by reduced proliferation or increased apoptosis, and that *Nfil3*-deficient fetal ILC precursors were unable to further mature in vitro. In addition, we found that *Nfil3*-dependent CHILP strongly upregulated NFIL3 expression in response to IL-7. Thus, we propose that cytokine-dependent signals may promote stabilization and/or enhancement of NFIL3, which in turn orchestrates the emergence of CHILP via direct *Id2* regulation.

Whereas our results clearly indicate an essential role for NFIL3 in early ILC precursors, NFIL3 may also play additional contextdependent roles at later stages of ILC differentiation and for maintenance of effector functions in mature ILC subsets. Recent studies of conditional ablation of *Nfil3* in NK cells and NCR⁺ ILC3 (using *Ncr1*-Cre mice) failed to identify a major role for NFIL3 in NK cell proliferation following MCMV infection and maintenance of mature ILC3, respectively (Firth et al., 2013; Geiger et al., 2014). Similarly, our results using *Rorc*-Cre mice likewise suggest that many critical functions associated with ILC3 subsets (LTi and lymphoid tissue organogenesis) are intact despite



Figure 6. NFIL3 Acts in the CHILP via Direct Control of Id2 Expression

(A) Nfi/3^{+/+} and Nfi/3^{-/-} FLT3⁻α4β7^{high} cells were analyzed by quantitative RT-PCR for *Id2* expression.
(B) Mean fluorescence intensity of *Id2*^{GFP} in E15.5 FL FLT3⁻α4β7^{high} cells from Nfi/3^{+/+}, Nfi/3^{+/-}, and Nfi/3^{-/-} littermate controls.

(C) Id2 locus scheme (top). ChIP analysis for NFIL3 and H3K4me2 binding in the Id2 locus of BM CHILP cells (left panel) and E15.5 CD4⁻ LTi cells (right panel). (c) Io vivo Id2 rescue scheme (top). Hematopoietic progenitor cells from *Mills*^{+/+} and *Mills*^{-/-} (CD45.2) mice were transduced with pMig.Id2-IRES-GFP retroviral vector and control, and 5 × 10⁵ total cells were injected into irradiated NSG hosts (CD45.1). GFP-positive cells were analyzed by flow cytometry, and cell numbers normalized to the transduction efficiency are displayed. Nfi/3+/+ pMig-Empty n = 4; Nfi/3+/+ pMig-Id2 n = 4; Nfi/3-/- pMig-Empty n = 4; Nfi/3-/- pMig-Id2 n = 5. (E) NFIL3 controls helper-like innate lymphoid cell generation through regulation of the CHILP.

Error bars show SE. * and ** p values for Student's t test (B), ANOVA test (C), and Mann-Whitney (D) lower than 0.05 and 0.01, respectively. See also Figure S6.

Nfil3 ablation after ILC subset commitment (Figure S2B). In addition, Nfil3-deficient ILC2 displayed normal cytokine secretion and expansion in response to IL-33 (Figures S5D-S5F). Finally, whereas NFIL3 binding to the Id2 locus was a common characteristic of ILC progenitors and mature ILC2 and ILC3, NFIL3 binding occurred at different Id2 genomic regions in the CHILP and mature ILC, suggesting differential transcriptional activity of NFIL3 in ILC progenitors and committed cells. Nevertheless, detailed studies on conditional Nfil3 deletion in mature ILC subsets need to be performed in order to fully address this question.

Our current data demonstrating that NFIL3 is a key regulator of the common-helper-like innate lymphoid precursor and previous studies establishing that cytotoxic ILC development, notably NK cells, also rely on NFIL3 suggest that NFIL3 may be required for the early establishment of a common helper- and cytotoxic-ILC

lineage progenitor (Figure 6E). In line with this idea, it was recently shown that NFIL3 can direct the development of a common cytotoxic and helper ILC precursor (Yu et al., 2014). Genetic fate-mapping studies, multiparametric reporter lines, and lineage-targeted strategies will be central to further elucidate the existence and the fate of such global innate lymphoid progenitor (GILP) to helper and cytotoxic ILCs (Figure 6E).

EXPERIMENTAL PROCEDURES

Mice

C57BI/6 mice were purchased from Charles River. Nfil3GFP transgenic mice were generated using bacterial artificial chromosomes (BACs) (obtained from Gene Expression Nervous System Atlas) that comprise the Nfil3 gene with upstream and downstream regulatory regions. NSG and C57BI/6 Ly5.1 (CD45.1) mice were maintained in house at Instituto de Medicina Molecular

(IMM) or purchased from The Jackson Laboratories. *Nfil3^{-/-}, Vav1*-iCre, *Rorc*-Cre, *Nfil3^{n/n}*, *Rorc*-GreP, and *Id2*^{GFP} mice were on a C57BI/6 background and were previously described (de Boer et al., 2003; Eberl and Littman, 2004; Eberl et al., 2004; Gascoyne et al., 2009). Motomura et al., 2011; Rawlins et al., 2009). Mice were analyzed at the age of 8-12 weeks. All animal experiments were approved by national and institutional ethical committees, Direção-Geral de Alimentação e Veterinária, and IMM and Institute Pasteur ethical committees. Power Analysis was performed in order to estimate the number of experimenta mice.

Flow Cytometry Analysis and Cell Sorting

Embryonic guts and lymph nodes were harvested and digested with collagenase D (5 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) in DMEM, 3% FBS for approximately 40 min at 37°C under gentle agitation. Fetal liver and LN cell suspensions were obtained using 70-µm strainers. Bone marrow cells were collected by either flushing or crushing bones. Lungs were minced and incubated 30 min at 37°C with agitation in HBSS with 5 mM EDTA, 10 mM HEPES, and 5% FBS followed by 1 hr digestion with collagenase D (5 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) in RPMI, 5% FBS with 10 mM HEPES. Sequentially cells were purified by centrifugation 30 min at 2,400 rpm in 40/80 Percoll (Sigma) gradient. Small intestines were cut, washed with PBS 1× 5 mM EDTA 15 min at 37°C with agitation. IELs were removed using a 100- μm cell strainer, and the remaining pieces were digested 30 min at 37°C with agitation in RPMI with 10 mM HEPES and 5% FBS, collagenase D (5 mg/ml; Boche) and DNase I (0.1 mg/ml; Boche). Sequentially, cells were purified by centrifugation 30 min at 2,400 rpm in 40/80 Percoll (Sigma) gradient. Livers were smashed and cells were purified by centrifugation 30 min at 2,400 rpm in 35% Percoll (Sigma). Intracellular stainings for transcription factors were performed using the Foxp3/ Transcription Factor Staining Buffer Set (eBioscience). For cytokine production, cells were stimulated for 4 hr with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (3 μ g/ml) and analyzed by intracellular staining as described (Klein Wolterink et al., 2013). The lineage cocktail for adult BM, liver, lung, and gut included CD3ε, CD4, CD8α, CD19, B220, CD11c, CD11b, Ter119, Gr1, TCRβ, TCRγδ, and NK1.1. For ILC1 staining in BM, liver, and gut, NK1.1 and CD11b were not added to the lineage cocktail. The lineage cocktail for FL, E15.5 guts, and LN included Ter119, TCRβ, CD3€, CD19, NK1.1, CD11c, CD11b, and Gr1. Samples were sorted on a FACSAria I or FACSAria III and analyzed on a LSRFortessa (BD). Flow cytometry data were analyzed with FlowJo 8.8.7 software (Tree Star). Sorted populations were >95% pure. A complete description of all populations analyzed and sorted is available in the Supplemental Experimental Procedures.

Bone Marrow Transplantation

BM cells were KIT⁺ MACS sorted from $Nfil3^{+/+}$ or $Nfil3^{-/-}$ mice. 5 × 10⁵ sorted cells were retro-orbitally injected in direct competition with a third-part WT competitor CD45.1/CD45.2 (1:1 ratio) into lethally irradiated NSG CD45.1 mice. Recipients were analyzed 8 weeks post-transplantation.

Cell Culture and Viral Transduction

For embryonic cell culture, ILC precursors and CD4⁻ LTi cells were sorted from E15.5 guts and suspended in culture medium OPTI-MEM (Invitrogen) supplemented with 20% FBS, penicillin and streptomycin (respectively, 50 U and 50 mg/ml; Invitrogen), and recombinant murine RANK ligand (rRANKL) (50 ng/ml; Peprotech). Cells were seeded into flat-bottom 96-well plates previously coated with 30,000 rad-irradiated OP9 stromal cells for 6 days. For IL-7 stimulation, cells were sorted and stimulated overnight (10 ng/ml), and all conditions were analyzed using a live/dead cellular marker. STAT5 inhibitor was purchased from Santa Cruz (sc-355979). For ILC2 differentiation, precursor cells were cultured on OP9-DL1 cells in the presence of FLT3 ligand (10 ng/ml), IL-7 (10 ng/ml), and acquisition of PLZF, CLPs were cultured 5 days on OP9-DL1 cells in the presence of FLT3 ligand (10 ng/ml), IL-7 (10 ng/ml), and the presence of FLT3 ligand (10 ng/ml), IL-7 (10 ng/ml), and SCF

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(10 ng/ml). For retroviral transduction, cells from *Nfil3^{-/-}* and WT littermate controls were sorted and transduced with pMig.IRES-GFP retroviral empty vector or containing *Id2* in the presence of polybrene (0.8 μ g/ml; Sigma-Aldrich).

Quantitative RT-PCR

Total RNA was extracted using RNeasy Micro kit (QIAGEN) according to manufacturer's protocol. RNA concentration was determined using Nanodrop Spectrophotometer (Nanodrop Technologies). For TaqMan assays (Applied Biosystems), RNA was retro-transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in real-time PCR. Gene expression was normalized to Hprt1 and Gapdh. When multiple endogenous controls are used, these are treated as a single population and the reference value calculated by arithmetic mean of their CT values. Thus, we used the comparative C_T method (2 ^{$-\Delta CT$}), in which ΔC_T (gene of interest) = C_T (gene of interest) C_{T} (Housekeeping reference value). When comparison or fold between samples was necessary, the comparative ΔC_T method (2 $^{-\Delta\Delta CT}$), in which $\Delta\Delta C_T$ (gene of interest, population of interest) = ΔC_T (gene of interest, population of interest) = ΔC_{T} (gene of interest, reference population). Real-time PCR analysis was performed using StepOne Real-Time PCR system (Applied Biosystems). Probes can be found in Supplemental Experimental Procedures.

ChIP Assay

BM CHILP, E15.5 CD4⁻LTi, BM ILC2, and gut ILC3 cells were isolated by flow cytometry sorting. Cells were lysed, crosslinked, and chromosomal DNA-protein complex sonicated to generate DNA fragments ranging from 100 to 300 bp. DNA/protein complexes were immunoprecipitated, using the LowCell# ChIP kit (Diagenode), with 3 µg of rabbit polyclonal antibody against NFIL3 (H-300; Santa Cruz Biotechnology), rabbit control IgG (Abcam), or H3K4me2 (07-030; Millipore). Immunoprecipitates were uncrosslinked and analyzed by quantitative PCR using primer pairs (5'-3') flanking putative NFIL3 sites on *Id2*. Results were normalized to input intensity and control IgG. Experimental controls also included NFIL3 chromatin immunoprecipitation (ChIP) in fetal *Nfil3*-deficient ILC3 (Figure S6C) and NFIL3 ChIP analysis in an irrelevant non-transcribed region (segment A; Figure 6C). Primer sequences are indicated in the Supplemental Experimental Procedures.

Statistics

Variance was analyzed using F-test. Student's t test was performed on homoscedastic populations, and Student's t test with Welch correction was applied on samples with different variances. Mann-Whitney *U* test was used in samples that did not follow a normal distribution. Equality of several means was determined by ANOVA test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/ 10.1016/j.celrep.2015.02.057.

AUTHOR CONTRIBUTIONS

W.X. designed, performed, and analyzed the experiments in Figures 1A–1C, 1E, 3C, 3F, 5A–5F, S1A, S1B, S3A, S5A, S5D, S5E, and S6E, R.G.D. designed, performed, and analyzed the experiments in Figures 1A, 1C, 1D, 1F, 1G, 2B–2H, 3A, 3B, 4A–4D, 6A–6C, S1C–S1I, S2A, S2B, S4A–S4E, and S6A–S6C. D.F.-P. designed, performed, and analyzed the experiments in Figures 1A, 1C–1F, 2A–2D, 3A–3F, 4A–4D, 6D, S3A, S3B, S4A–S4E, S5B, S5C, and S6A. C.V. generated and characterized *Nfil3*^{GFP} transgenic mice. M.F. and Y.M. designed, performed, and analyzed experiments; H.R., S.L.-L., L.M.-S., and F.B. contributed to several experiments; V.B., B.K., H.B., M.C.C., and M.K. designed experiments; and J.P.D. and H.V.-F. directed the study and wrote the manuscript.

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Synthesis (french)

Les cellules souches hématopoïétiques (CSH) sont responsables de la formation de trillions de cellules sanguines chaque jour chez l'adulte. D'une part, les CSHs ont la capacité de donner naissance à des cellules-filles CSH non différentiées ; de l'autre les CSHs peuvent entrer dans un processus de différentiation dynamique et bien organisé qui génère d'autres cellules filles. Cette perte graduelle du potentiel de différentiation est modulée par l'expression de facteurs de transcription qui, en fin de compte, guide le développement des différentes lignées du système immunitaire.

Le système immunitaire adaptatif désigne deux larges groupes de cellules qui réagissent aux antigènes : les lymphocytes B (de Bursal ou dérivé du "bone marrow") et les lymphocytes T (dérivé du thymus), identifiés respectivement par les protéines de surface CD19 et CD3. En l'absence de stimulation, les cellules naïves expriment un ample répertoire d'antigènes (Ag). Alors qu'ils rencontrent leur antigène cible, les lymphocytes portant le récepteur correspondant prolifèrent massivement formant des clones qui ensuite deviennent les effecteurs de la réponse immunitaire adaptative. Après l'élimination de l'antigène, une fraction de cellules « mémoire » spécifiques d'antigène circulent et « patrouillent » l'organisme pendant des années jouant un rôle central dans la réponse immunitaire secondaire contre le même antigène (caractéristique principale de l'immunité adaptative). La maturation des lymphocytes T s'effectue au sein du thymus et consiste entre autre en l'expression d'un TCR (récepteur membranaire caractéristique des lymphocytes T) qui peut être constitué soit par des chaines gamma et delta (cellules $\gamma\delta$ -T), soit par des chaines alpha et beta (cellules $\alpha\beta$ -T). Les premiers sont moins bien compris et ont des caractéristiques à cheval entre les cellules innées et adaptatives. L'hétérogénéité de leur répertoire est beaucoup moins riche et ils sont localisés dans des sites très précis, comme dans la peau ou les surfaces muqueuses.

Contrairement à l'immunité adaptative, l'immunité innée est apparue déjà dans les premiers organismes multicellulaires et elle reconnait sa cible par des mécanismes à large spectre capables de détecter des motifs structurels conservés par des grands groupes de pathogènes, comme les lipopolysaccharides (LPS).

Depuis la découverte de la première cellule innée par Elie Metchnikoff, le macrophage, de nombreux acteurs de la réponse cellulaire innée ont été décrits. Du coté myéloïde on distingue deux classes : les cellules mononuclées (monocytes, macrophages et cellules dendritiques, CD ou DC en anglais) et les granulocytes (neutrophiles, éosinophiles, mastocytes et basophiles). La branche lymphoïde innée a été décrite plus récemment et est constituée par les cellules innées lymphoïdes (CLIs) et deux populations particulières de

lymphocytes qui répondent rapidement aux antigènes de manière non-spécifique, les cellules $\gamma\delta T$, discutés antérieurement et les cellules B-1.

Parmi les cellules dendritiques localisées dans le sang, la rate et organes lymphoïdes (dépourvues de marqueurs de lignée et exprimant le marqueur HLA-DR) on distingue deux grands raison de différences types en morphologiques, phénotypiques et fonctionnelles : les CD conventionnelles, peuvent être migratoires qui ou résidentes, (cCD) et les CD plasmacytoïdes (pCD). Les CD plasmacytoïdes, grâce à différents récepteurs (CD123, CD303 ou CD304), sont capables de capturer des





antigènes viraux, tumoraux ou issus de cellules en nécrose. L'étude de l'expression de leurs « toll-like receptors » TLR (TLR7 et 9) et des cytokines produites suite à leur engagement par leurs ligands respectifs, suggère une spécialisation fonctionnelle des pCD chez l'homme comme chez la souris dans les réponses anti-virales, nécessitant la production d'IFN-alpha, beta et lambda.

Les CD conventionnelles sont majoritaires dans le sang et se divisent en deux sousfamilles principales: les CD1c⁺ CDs qui co-expriment CD11b et de hauts niveaux de CD11c et les CD141⁺ cCDs qui ont une expression de CD11c plus faible et n 'expriment pas CD11b. Ces deux classes de CDs expriment aussi différemment les TLRs, alors que les CD1c⁺ CDs expriment tous les TLRs sauf TLR9, les CD141⁺ CDs expriment faiblement TLR1-2, TLR6 et TLR8 et fortement TLR3 et TLR1.

Les CDs ont traditionnellement eu comme caractéristique de se différencier à partir de deux types de précurseurs, un myéloïde et un lymphoïde. La recherche d'un précurseur commun aux CDs a récemment conduit à repenser l'architecture générale de l'hématopoïèse après qu'un précurseur commun aux CD, monocytes et macrophages : le MDP (macrophage dendritic cell precursor) ait été indentifié dans la lignée myéloïde. Ce MDP est capable de donner : soit les monocytes, macrophages et cellules de Langerhans, soit les pCD et les cCD à partir d'un précurseur commun aux CD : le CDP.



Figure 2: Schéma du développement des cellules dendritiques humaines. GMDP: granulocyte, monocyte and dendritic cell (DC) progenitor; CDP: common DC progenitor; MDP: monocyte-DC progenitor; pre-cDC: commited precursor of classical DCs.

Les cellules innées lymphoïdes (CLI pour innate lymphoid cells) forment une famille d'effecteurs de la réponse innée dépourvus de récepteurs spécifiques aux antigènes, dont la diversité fonctionnelle est proche de celle des effecteurs T helper (Th) de la réponse adaptative. On distingue trois principaux groupes d'CLI. Les CLI de type 1, constituées des cellules tueuses naturelles (de l'anglais natural killer, NK) identifiées il y a plus de 40 ans et les « helper » CLI1s qui produisent préférentiellement des cytokines de type 1, comme l'IFN gamma. Les CLI de type 2, productrices d'IL-13, d'IL-5 et d'IL-4, comme les cellules Th2, interviennent dans la réponse innée mucosale aux parasites intestinaux, et participent à l'exacerbation des réactions inflammatoires et allergiques des voies respiratoires. Les CLI de type 3 se distinguent par l'expression du facteur de transcription RORyt (RAR-related orphan

du receptor gamma t) et récepteur de l'IL-23, qui leur confèrent la capacité de sécréter de l'IL-17 et de l'IL-22, au même titre que les cellules Th17.. Présentes dès le stade fœtal, elles sont indispensables à la formation des ganglions périphériques et des tissus lymphoïdes associés à l'intestin. Après la naissance, elles contribuent à protéger la muqueuse contre les entérobactéries pathogènes, et à maintenir la flore commensale sous contrôle.



Les modèles animaux ont largement contribué à notre compréhension de l'immunologie humaine et des mécanismes pathologiques associés au développement des maladies. Cependant, les modèles murins ne permettent pas de reproduire toute la complexité du system immunitaire humain et des pathologies humaines. La sélection et la validation de nouvelles thérapies et vaccins sont devenues incontournables dans les domaines de l'immunothérapie des cancers, de l'infectiologie et des maladies auto-immunes. Récemment, les avancements dans la reconstitution d'un système immunitaire humain dans des modèles murins immunodéficients « HIS » (Human Immune System), a relancé l'intérêt de l'utilisation de tels modèles pour résoudre les mécanismes physiopathologiques de maladies liées à l'immunité et pour prédire les réponses immunitaires aux cours des traitements de maladies complexes.

Le développement des souris humanisées a débuté à la fin des années 1980 avec des études réalisées dans des souris immunodéficientes portant la mutation SCID (Severe Combined Immunodeficiency Desease). Depuis, nombreux efforts ont été déployés pour atteindre un modèle dont la greffe et le développement du système immunitaire humain sont optimaux, en modifiant le fond génétique et en mutant des gènes nécessaires au développement des cellules murines.



Figure 4: Stratégies d'amélioration de l'homéostasie et fonction des cellules du système immunitaire humain chez les souris HIS a travers le temps

Dans les travaux présentés dans ce manuscrit, le modèle BRGS (BALB/c Rag2^{-/-} Il2rg^{-/-} NOD.Sirpa) a été utilisé. Les souris BRGS ont un fond Balb/c et présentent des mutations nulles sur le gène RAG-2, (Recombinase Activity Gene-2) qui empêche le développement des lymphocytes T, B et la génération d'anticorps, et sur le gène de la chaine γ c (CD132), commune à plusieurs récepteurs d'interleukines (IL-2, IL-4, IL-7, IL-9, IL-15 et IL-21), empêchant le développement des cellules Natural Killer (NK) et autres cellules innées. Enfin, l'insertion de la version polymorphique du gène SIRP α (Signal regulatory protein alpha) du fond NOD confère une tolérance supérieure des phagocytes vers les cellules humaines portant le antigène CD47, et ainsi une meilleure reconstitution hématopoïétique.

Ces modifications rendent les souris BRGS profondément immunodéficientes car dépourvues de lymphocytes T, de lymphocytes B et de cellules NK et, par la suite, favorisent la greffe et la différenciation des Cellules Souches Hématopoïétiques humaines contenues dans la fraction de cellules CD34+ (CSHh) de la moelle osseuse, du sang de cordon ou du foie fœtal. Après greffe de ces dernières, le développement des différentes lignées immunitaires humaines est observé pendant plusieurs mois.

Ainsi, les modèles BRGS sont un outil préclinique particulièrement approprié permettant d'obtenir de manière rapide des résultats pertinents d'un point de vue clinique. Cependant, certains types cellulaires, comme les cellules dendritiques ou le cellues lymphoïdes innées, ne parviennent pas à se différencier et à fonctionner normalement dans les modèles murins HIS actuels.

Alors que les cellules dendritiques sont essentielles pour l'initiation de la réponse immunitaire et la présentation antigénique, ils agissent aussi de concert avec les cellules de l'immunité innée (notamment les CLIs) pour réguler une réponse optimale et, à long terme, la réponse humorale et l'immunité cellulaire. Alors que les modèles HIS génèrent un répertoire complet de cellules T et B, ces modèles ont des réponses atténuées après une primo immunisation. Ceci peut être dû à une génération sous-optimale des cellules innées myéloïdes et lymphoïdes « de réponse rapide ». Dans le cadre de ce projet, ces défauts de réponse immunitaire seront corrigés par différentes approches innovantes, incluant l'élimination de la compétition des cellules dendritiques murines (par élimination du facteur de croissance murin flk2) ou par injection de facteurs de croissance humains pour stimuler le développement des cellules dendritiques et/ou CLIs (injection de Flt3 et IL-15).

Ces études ont comme objectifs spécifiques : Premièrement, créer et valider un modèle de souris HIS permettant de récapituler le phénotype et les fonctions des CLIs humaines. Deuxièmement, comprendre comment ces cellules CLI se développent in vivo chez les souris humanisées. Finalement, optimiser l'utilisation de modèles HIS comme outil de test préclinique de immunothérapies contre le cancer.

Je présente les résultats principaux de mes travaux de thèse sous forme d'articles scientifiques :

Des interactions functionnelless avec les CDs promeuvent l'homeostasie des CLIs in vivo.

L'ingénierie génétique a permis de décrire l'ontogénie des CLIs murins au point qu'elle est aujourd'hui relativement bien connue. En revanche, le développement des CLIs chez l'homme reste largement méconnu du fait de la grande variété des précurseurs et des conditions de cultures utilisées dans les études publiées. Pour concilier ces modèles différents et pour étudier la diversité phénotypique et fonctionnelle des CLIs, nous visons à créer des modèles in vivo récapitulant les CLIs humains avec fidélité. De plus, ces modèles peuvent finalement être utilisés pour recréer des pathologies humaines et pour étudier le potentiel des CLIs comme cible thérapeutique ou vaccinale.

La différentiation des CLIs implique de nombreuses interactions entre des cellules précurseurs, leur stroma et les cellules myéloïdes, ainsi que les voies de signalisation impliquant les facteurs solubles que ces partenaires cellulaires produisent. Cependant, les cellules myéloïdes humaines ne sont pas suffisamment représentées dans les souris HIS du fait de l'absence de cytokines humaines et à la compétition pour la niche hématopoïétique dans les tissue murins. Une de ces cytokines essentielles pour le développement myéloïde est le Flt3L (de l'anglais FMS-related tyrosine kinase 3 ligand).

Dans cette étude, nous avons généré des souris HIS BALB/c Rag2^{-/-}Il2rg^{-/-} Sirpa^{NOD}Flk2^{-/-} (BRGSF) qui, après traitement avec la protéine recombinant Flt3L, ont montré une augmentation des CDs humaines. Cette amélioration a eu, par la même occasion, un impact positif sur l'homéostasie et la fonction des CLIs humaines.

Nous montrons dans ces travaux que l'interaction CD-CLI a lieu au sein de tous les organes lymphoïdes et non-lymphoïdes (muqueuses), permettant aux CLIs de se développer et survivre grâce à des facteurs solubles (interactions indirectes) et probablement à des interactions directes. Sur cette base, les souris HIS BRGSF ouvrent un vaste champs de recherche pour l'étude du développement des CLIs, des pathologies associées, et de nouvelles stratégies thérapeutiques.



Figure 5: Resumé graphique du modéle de souris humanisée BRGSF.

Des cellules precurseurs des CLI fournissent un substrat pour la differentiation des CLIs dans les tissus.

Les CLIs peuvent être identifiés par les marqueurs lignage⁻⁻CD7⁺CD56⁻⁻ CD127⁺ à basse fréquence dans le sang périphérique des individus sains ou souffrant des divers syndromes cliniques. Une analyse plus approfondie utilisant d'autres marqueurs peut

les séparer en trois grandes classes. Jusqu'à présent, la manière dont les cellules précurseurs des CLI (CLIP) donnent naissance à différentes CLI matures dans les tissus restait mal comprise. Cette étude collaborative au sein de mon équipe a donné lieu à la découverte des CLIP circulants dans le sang périphérique humain.

La mise en évidence des CLIP circulantes et la possibilité de les prélever permettent d'envisager avec optimisme des thérapie applications de cellulaire. Ces précurseurs donnent en effet naissance à quatre grands types de cellules lymphoïdes innées qui interviennent très défendre tôt pour l'organisme : les cellules tueuses naturelles



Figure6: Resumé graphique de la localization et capacité de differentiation des CLIPs.

(NK), et les trois types d'CLI (CLI1, 2 ou 3), qui possèdent des rôles et des cibles différents. Cette découverte bouscule également le dogme couramment admis jusqu'à présent sur l'origine tissulaire des CLI (c'est à dire la moelle osseuse). Les CLIP circulent dans le sang puis parviennent sur le lieu de l'infection ou de la tumeur, où les CLI sont produites localement pour combattre les agressions. La production des CLI est donc locale, dans chaque tissu concerné par un mécanisme qu'on pourrait nommer l'CLI-poïèse, à l'image d'autres processus de fabrication cellulaire dans l'organisme. »

Dans cette étude on a utilisé des souris HIS BALB/c Rag2^{-/-}Il2rg^{-/-}Sirpa^{NOD} (BRGS) pour analyser le potentiel de differentiation de la population cellulaire CD117⁺ CLIP. Alors que les souris BRGS greffées avec des CSH ont montré la reconstitution de plusieurs lignées cellulaires, celles qui ont recu les cellules CD117⁺ CLIP ont uniquement montré le developpement des cellules Lin⁻CD7⁺ mais pas des cellules myeloides, lymphocytes B ou
lymphocytes T. La progénie humaine dérivée des CD117⁺ CLIP a été detectée dans plusieurs organes (rate, poumon, intestin et foie). Dans tous ces tissus, des cellules EOMES⁺ NK ainsi que des populations cellulaires CD127⁺ CLI productrices de IFN- γ ont pu être détectées.

Le precurseur CLI a pu être identifié également dans les souris BRGS greffées avec des CSHs CD34⁺. De facon similaire aux CLIPs obtenues à partir du sang humain, les cellules derivées des souris humanisées ont echoué a produire des cytokines après stimulation ex vivo. Toutefois, la culture de ces cellules in vitro en presence de IL-2, IL-7 et IL-1 β a conduit à l'expansion cellulaire et à la production de IFN- γ , IL-13, IL-17A et IL-22, démontrant donc la présence des CLIPs dans les souris HIS.

Les cellules tueuses naturelles derivées des cellules souches du sang de cordon ombilical (UCB) sont efficaces dans le traitement in vivo du cancer colorectal metastatique.

Le cancer colorectal (CRC) est la quatrième cause de mortalité par cancer dans le monde. Malgré les derniers avancements dans le traitement du CRC métastasique qui ont amélioré substantiellement le taux de survie, cette maladie est encore souvent fatale. Les anticorps monoclonaux (mAbs) ciblant le récepteur du facteur de croissance épidermique (EGFR de l'anglais Epidermal Growth Factor Receptor) ont été approuvés pour le traitement des patients qui souffrent de CRC avancé, soit en combinaison avec la chimiothérapie ou comme monothérapie (dans les patients chimio-réfractaires). Cependant, des mutations sur des gènes suppresseurs de tumeurs et proto-oncogènes dans la voie de signalisation du EGFR (RAS, BRAF ou PIK3CA) son fréquentes chez les patients avec CRC. Ces mutations représentent des marqueurs de pronostic négatifs et rendent les thérapies mAbs anti-EGFR inefficaces. Cela se traduit par le fait que 42% des patients avec CRC chimio-réfractaires sont dépourvus d'options thérapeutiques.

En plus du blocage de l'interaction ligand-EGFR dans les cellules tumorales, les mAbs thérapeutiques peuvent aussi interagir avec les cellules NK, déclenchant une cytotoxicité dépendante des anticorps (ADCC, de l'anglais antibody-dependent cell-mediated cytotoxicity). Plusieurs études ont montré un phénotype dysfonctionnel et une infiltration déficiente des NKs dans le tissu cancéreux dès les stades précoces ainsi qu'un microenvironnement tumoral immunosuppresseur. Différentes stratégies, comme l'infusion des NK autologues, ont été utilisées pour augmenter l'activité anti-tumorale des cellules NK,

mais elles se sont relevées jusqu'à présent infructueuses dans plusieurs types de tumeurs solides.

Dans cette étude nous avons utilisé des souris BRGS pour évaluer le potentiel thérapeutique d'un nouveau traitement combinatoire dans un modèle de CRC muté pour RAS (RAS^{mut}) et exprimant l'EGFR. Ce traitement a consisté en une co-administration de cellules NK dérivées des cellules souches d'UCB et de mAb commercial cetuximab. Nos résultats montrent une efficacité anti-tumorale des cellules UCB-NK contre les cellules tumorales EGFR⁺ RAS^{mut} supérieur au cetuximab en monothérapie. Cette activité anti-tumorale a été observée à la fois in vitro et in vivo contre des cellules cancéreuses colorectales indépendamment du statut EGFR et RAS.



Figure 7: Effet des differents traitements sur la croissance de la tumeur SW480 chez les souris BRGS.

Une approche basée sur des nanobodies bispecifiques pour exploiter la capacité cytolytique des cellules $V\gamma 9V\delta 2$ -T

Comme discuté précédemment, les cellules T dont le TCR est composé par des chaines de type γ et δ , particulièrement les cellules V γ 9V δ 2-T, présentent un intérêt particulier pour développer de nouvelles approches thérapeutiques. En fait, des essais cliniques ont récemment débuté pour des thérapies utilisant ces cellules dans des cancers hématologiques ou solides. Ces stratégies incluent le transfert adoptif des cellules V γ 9V δ 2-T amplifiées ex vivo et l'activation in vivo des cellules V γ 9V δ 2-T à travers l'administration d'aminobisphosphonates ou phosphoantigènes synthétiques seuls ou combinés avec IL-2. Cependant, les résultats de ces essais montrent une tolérance et une activité anti-tumorale qui varient selon les études. Une explication possible pour cette observation est le fait que

l'induction de l'activité des cellules V γ 9V δ 2-T est systémique et non favorisée dans le microenvironnement tumoral, où les cellules effectrices doivent exercer leur activité.

Des travaux publiés récemment ont montré que les nanobodies (ou VHHs) peuvent améliorer l'activation et l'accumulation des cellules V γ 9V δ 2-T dans la tumeur. Les nanobodies sont des fragments d'anticorps à domaine unique plus de dix fois plus petits que les anticorps monoclonaux traditionnels. Les nanobodies se caractérisent par d'étonnantes propriétés en termes de production, de stabilité et de reconnaissance de marqueurs tumoraux. Leur petite taille permet entre autres de cibler la même protéine de façon multiple.

Dans cette étude nous décrivons la génération de nanobodies bispécifiques qui combinent l'inhibition de la voie de signalisation de l'EGFR et l'activation spécifique des

cellules effectrices Vy9Vô2-T. Un modèle BRGS de carcinome colorectal a été utilisé pour évaluer l'efficacité in vivo de cette stratégie thérapeutique. Nous avons pu démontrer que les cellules Vy9V82-T activées de cette façon produisent des cytokines pro-inflammatoires comme IFN- γ et TNF- α in vitro et in vivo indépendamment du statut du gène KRAS. Cette activité anti-tumorale a conduit à une charge tumorale diminuée et une survie accrue des souris traitées en comparaison à celles qui n'ont pas reçu le traitement combiné de cellules V γ 9V δ 2-T et de nanobodies.



Figure8: Effet des différents traitements sur la croissance de la tumeur SW480 (c) et sur la survie des souris BRGS (d).

LIST OF PUBLICATIONS

ORIGINAL ARTICLES

Human thymopoiesis is controlled by a genetic variant within the *TCRA-TRCD* locus. Emmanuel Clave, Itauá Leston Araujo, Cécile Alanio, Etienne Patin, Jacob Bergstedt, Alejandra Urrutia, <u>Silvia Lopez-Lastra</u>, Yan Li Bruno Charbit, Cameron Ross MacPherson, Milena Hasan, Breno Luiz Melo-Lima, Noemie Saut, Marine Germain, David-Alexandre Tregouet, Pierre-Emmanuel Morange, Magnus Fontes, Darragh Duffy, James Philip Di Santo, Lluis Quintana-Murci, Matthew L. Albert, Antoine Toubert, for The Milieu Intérieur Consortium. Science Translational Medicine (2017). *Under revision*.

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Regulatory T cells modulate high-dose IL-2-induced toxicity: modeling IL-2 immunotherapy in human immune system mice. Yan Li, Jiazi Ren, Ai-ing Lim, Helene Strick-Marchand, Guillemette Masse-Ranson, <u>Silvia Lopez-Lastra</u>, Dan Li, Gregory Jouvion, Bin Li and James P. Di Santo. Submitted. **NFIL3** orchestrates the emergence of common helper innate lymphoid cell precursors. Wei Xu, Rita G. Domingues, Diogo Fonseca-Pereira, Manuela Ferreira, Helder Ribeiro, <u>Silvia Lopez-Lastra</u>, Yasutaka Motomura, Lara Moreira-Santos, Franck Bihl, Veronique Braud, Barbara Kee, Hugh Brady, Mark C. Coles, Christian Vosshenrich, Masato Kubo, James P. Di Santo and Henrique Veiga-Fernandes. Cell Reports 2015. 10; 12 : 2043 - 2054.

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REVIEW ARTICLES

NK Cell Development in Human Immune System (HIS) Mice and Their Role in HIV Pathogenesis. <u>Silvia Lopez-Lastra</u>, Yan Li, Guillemette X. Masse and James P. Di Santo. Springer Books 2014. 14:1

Modeling Natural Killer Cell Targeted Immunotherapies. <u>Silvia Lopez-Lastra</u> & James P. Di Santo. Front Immunol. 2017; 8: 370.

Titre : LES SOURIS HUMANISÉES COMME MODÈLES D'ÉTUDE DE L'IMMUNITÉ INNÉE HUMAINE ET DES IMMUNOTHERAPIES

Mots clés : Souris humanisées, Cellules Natural Killer, Cellules lymphoïdes innées, Immunothérapies

Résumé : Les modèles animaux ont largement contribué à notre compréhension de l'immunologie humaine et des mécanismes pathologiques associés au développement des maladies. Cependant, les modèles murins ne permettent pas de reproduire toute la complexité des pathologies humaines. Les souris à système immunitaire humain (HIS), par leur capacité à récapituler l'hématopoïèse humaine et à être infectées par des pathogènes humains, constituent une solution de choix pour combler ce fossé inter-espèce.

Après greffe de cellules souches hématopoïétiques humaines, des souris hôtes sévèrement immunodéprimées permettent un haut niveau de développement du système hémato-lymphoïde humain tout au long de leur vie. Cependant, certains types cellulaires, comme les cellules lymphoïdes innées, ne parviennent pas à se différencier et à fonctionner normalement dans les modèles murins HIS actuels. Ici, nous décrivons le développement d'un modèle souris HIS original. nommé BRGSF (BALB/c $Rag2^{tm1Fwa}Il2rg^{tm1Cgn}Flk2^{tm1lrl}$ Sirpa^{NOD}), montrant une amélioration de la maturation, de la fonction et de l'homéostasie des cellules natural killer (NK) humaines et des autres ILCS.

De plus, en récapitulant les différentes étapes du développement des ILCs humaines, ce modèle souris

BRGSF nous a permis d'identifier pour la première fois un précurseur d'ILC (ILCP) présent à la fois dans notre modèle HIS ainsi que dans le sang périphérique et plusieurs organes lymphoïdes et non-lymphoïdes humains. Cette population circulante d'ILCPs pourrait constituer un substrat pour la production d'ILCs matures dans les tissus périphériques en réponse à des stress environnementaux, inflammatoires et/ou infectieux.

Dans une seconde partie de ce travail de thèse, nous avons utilisé ces souris BRGS afin de tester l'efficacité de deux immunothérapies reposant sur les lymphocytes innés pour le traitement d'un carcinome colorectal exprimant EGFR et muté pour KRAS. La première approche a consisté en la co-administration des cellules NK dérivées de sang de cordon ombilical et d'anticorps monoclonal cetuximab afin de promouvoir le mécanisme de cytotoxicité cellulaire dépendante des anticorps (ADCC) contre la tumeur. La seconde stratégie a reposé sur l'injection de nanobodies VHH combinant l'inhibition de l'EGFR et l'activation spécifique du récepteur V γ 9V δ 2 des cellules T effectrices.

Les résultats de cette étude soulignent l'importance des modèles murins HIS pour la compréhension du développement des lymphocytes innés humains et pour mieux les mettre à profit dans les thérapies anti-tumeurs

Title : HUMANIZED MICE AS MODELS TO STUDY HUMAN INNATE IMMUNITY AND IMMUNOTHERAPIES

Keywords : Humanized mice, Natural killer cells, Innate lymphoid cells, Immunotherapies

Abstract : Animal models have extensively contributed to our understanding of human immunobiology and to uncover the underlying pathological mechanisms occurring in the development of the disease. However, mouse models do not always reproduce the genetic complexity inherent in human disease conditions. Human immune system (HIS) mouse models that are susceptible to human pathogens and can recapitulate human hematopoiesis provide one means to bridge the interspecies gap.

Severely immunodeficient host mice support lifelong, high level human hematolymphoid development after engraftment with human hematopoietic stem cells (HSC). However, the differentiation and function of some blood cell types, including innate lymphoid cells (ILCs), is poorly characterized in current HIS mice. Here we describe the development of a novel HIS mouse model, named BRGSF (BALB/c Rag2^{tm1Fwa}II2rg^{tm1Cgn}Flk2^{tm1Irl}Sirpa^{NOD}), which demonstrate enhanced maturation, function and homeostasis of human natural killer (NK) cells and other ILCs.

Furthermore, the BRGSF-based HIS mouse model recapitulated the developmental stages of human ILCs.

Furthermore, the BRGSF-based HIS mouse model recapitulated the developmental stages of human ILCs. We

could identify for the first time an ILC precursor (ILCP) population that is present both in HIS mice and in human peripheral blood as well as in several lymphoid and non-lymphoid human tissues. This circulating human ILCP population may provide a substrate to generate mature ILCs in tissues in response to environmental stressors, inflammation and infection.

In a second part of the thesis we used BRGS $(BALB/cRag2^{tm1Fwa}Il2rg^{tm1Cgnl}Sirpa^{NOD})$ immunodeficient innate lymphocyte-based mice to assess two immunotherapeutic approaches for treating EGFRexpressing KRAS-mutated colorectal carcinoma in vivo. The first model used a combination of umbilical cord blood (UCB)-derived NK cells and the monoclonal antibody cetuximab to promote antibody dependent cell cytotoxicity (ADCC) against the tumors. In a second model, we evaluated the therapeutic suitability of novel bispecific VHH constructs that combine inhibition of the EGFR with the target-specific activation of effector V γ 9V δ 2-T cells.

These studies highlight the utility for HIS-based mouse models to understand human innate lymphocyte development and to harness these potent effectors for antitumor therapies.