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Function and plasticity of NKp46 expressing innate lymphoid cells

Thomas Verrier

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Thomas Verrier. Function and plasticity of NKp46 expressing innate lymphoid cells. Human health and pathology. Université Sorbonne Paris Cité, 2016. English. NNT : 2016USPCC173 . tel-01743809

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Thèse de doctorat
de l'Université Sorbonne Paris Cité
Préparée à l'Université Paris Diderot
Ecole doctorale Bio-SPC (ED N°157)
Institut Pasteur Paris - Unité d'Immunité Innée

Function and Plasticity of NKp46 expressing Innate Lymphoid Cells

Thomas Verrier

Thèse de doctorat d'immunologie

Dirigée par James P. Di Santo

Présentée et soutenue publiquement à l'Institut Pasteur (Paris) le 30 Septembre 2016

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Acknowledgment

JDS Thesis Director – Constant support, Scientific guidance, Think Wider, Mentor For Life.
« FOCUS »

CV Supervisor – Scientific Advice, Diplomacy, Righteousness, FACS master.
« Sometimes the master has to do it himself »

NaS Supervisor – Energy & Joyfullness, My Sensei, Gut Princess, Mucosal Immunology Teacher.
« Bébé Thomas »

NiS Colleague – Everyday help, R&R lover, good jokes, intelligent design, Gut-prep professional
« For instance »

SM Colleague – Countless cigarett, strenght, determination, helpfulness, GOT addict
« Cersei va brûler port-royal avec le feu grejoy »

SLL PhD – Side by Side, La Combatienta Humanizada, Restless Queen of QUEEN
« Chewbacca »

AIL PhD – Will & Pure Sweetness, Science Machine, RUN RUN RUN
« Resilience »

GB Post-Doc – Belladonna, Fitness, Residence craver
« Fegato di ferro »

YL Post-doc – Transitional otaku, Hydrodynamic injection Titan, Undisturbed
« Be the opposite of yourself and see the light ?»

Innate Immunity Lab – No space, No time, Just love

AS / LBM / MD / NLL / DC / LS / HSM / GMR / PG / AC / JMD / SD / EF / HB / CP

Others know.

Special thanks : Ga. Light.

M/D/B/B/S

Note to the reader :

This manuscript presents my personal interpretations of complex biological system. My assumptions rely on selected scientific publications and extended bibliographic research, which is by no mean exclusive, and might become outdated in view of new discoveries.

Abbreviation List

AhR	Aryl hydrocarbon Receptor
AHR	Airway Hyper Responsiveness
AMP	Antimicrobial peptide
BM	Bone Marrow
BMDC	Bone Marrow derived Dendritic Cell
CBFB	Core Binding Factor β
CCR	« CC » Chemokine Receptor
CD	Cluster of Differentiation
CEC	Colonic Epithelial Cell
CHILP	Common Helper-like ILC progenitor
CLP	Common Lymphoid Progenitor
CYP	Cytochrome p450
CX3CR1	« CX3C » Chemokine Receptor 1
CXCL	« CXC » Chemokine Ligand
CXCR	« CXC » Chemokine Receptor
DC	Dendritic Cells
DN	Double Negative
DP	Double Positive
DR	Death Receptor
DSS	Dextran Sodium Sulfate
E4BP4	E4 promoter Binding Protein 4
EILP	Early ILC Progenitor
ER	Endoplasmic Reticulum
FL	Fetal Liver
Flt3	Fms-like Tyrosine Kinase 3
Fut2	Fucosyltransferase
GFP	Green Fluorescent Protein
GM-CSF	Granulocytes Macrophages Colony Stimulating Factor
HA	Hemagglutinin
HEK-293T	Human Embryonic Kidney 293 T cells
HFD	High Fat Diet
HMG	High Mobility Group
HSC	Hematopoietic Stem Cells
IDO1	Indoleamine 2,3- dioxygenase
IFN α	IFN α Receptor
IFNGR	Interferon γ Receptor
IL	Interleukin
ILC	Innate Lymphoid cells
kD	kiloDalton
KLRG1	Killer-cell Lectin-like Receptor subfamily G
KO	Knock-out
Lck	Lymphocyte Specific protein tyrosine kinase
Lin	Lineage
LN	Lymph Nodes
LSK	Lin ⁻ Sca-1 ⁺ c-Kit ⁺

LT	Lymphotoxin
LTi	Lymphoid Tissue inducer
MCMV	Mouse Cytomegalovirus
mLN	mesenteric Lymph Nodes
MHC	Major Histocompatibility Complex
Ncr1	Natural Cytotoxicity Receptor 1
Nfi3	Nuclear Factor Interleukin 3
NK	Natural Killer
NR	Nuclear Receptors
PLZF	Promyelocytic Leukemia Zinc Finger
PMA	Phorbol 12-Myristate 13-Acetate
PP	Peyer's Patches
RA	Retinoic Acid
RAG	Recombination Activating Gene
ROR	Retinoic Acid-related orphan receptor
S1pr5	Sphingosine 1-phosphate receptor 5
SCID	Severe Combined Immunodeficiency Syndrome
SG	Salivary Glands
SLO	Secondary Lymphoid Organs
SP	Single Positive
TCF-1	T-cell Factor 1
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TL1A	Tumor Necrosis Factor-Like Ligand 1A
TLR	Toll Like Receptor
TRAIL	Tumor Necrosis Factor Related Apoptosis-inducing Ligand
TSLP	Thymic Stromal-derived Lymphopietin
SCF	Stem Cell Factor
Sca-1	Stem Cell Antigen 1
WT	Wild Type
XCL1	« Xc » Chemokine Ligand

Cell population
 Miscellaneous
 Surface molecule
 Transcription factor
 Secreted molecule

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Introduction : A problem of nomenclature

Innate lymphoid cells (ILC) are critical immune effectors the early phases of immune responses (Fig.1). These RAG independent immune effectors respond to external stimuli solely through germ-line encoded surface receptors, which trigger their rapid cytokine production. ILCs originate from an Id2-dependent precursor and are separated in three groups reminiscent of helper T (T^H) cells. T^H1 cells and Group 1 ILC (ILC1) participate in the defense against intracellular pathogens, T^H2 cells and group 2 ILC (ILC2) contribute to the establishment of allergic responses and promote the expulsion of intestinal parasite while T^H3 cells and Group 3 ILC (ILC3) promote mucosal homeostasis, anti-fungal and anti-bacterial responses.

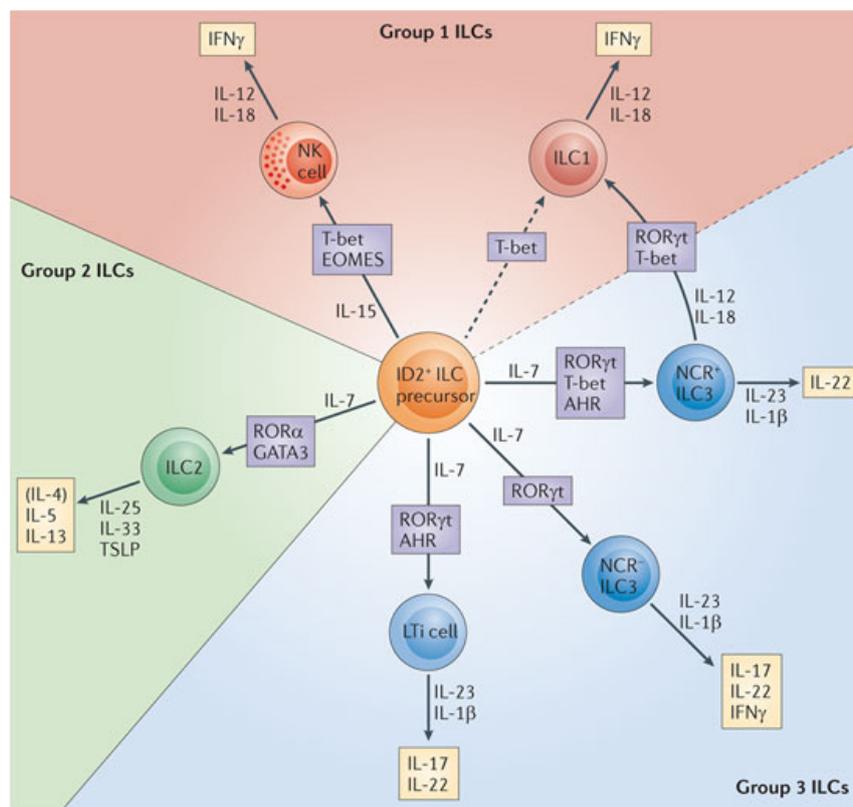
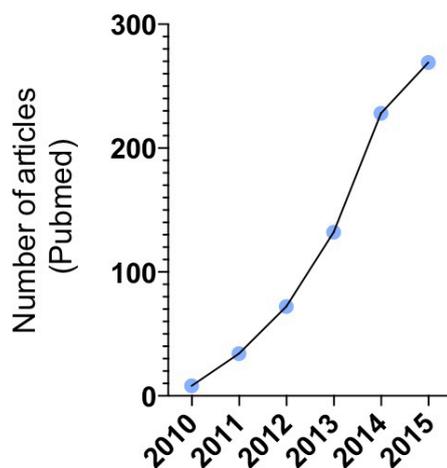


Figure 1 : Overview of the different ILC group
“Innate lymphoid cells – a proposal for uniform nomenclature”

Hergen Spits, David Artis, Marco Colonna, Andreas Diefenbach, James P. D Nature Reviews Immunology 13, 145-149 (February 2013) | doi:10.1038/nri3365 | Santo, Gerard Eberl, Shigeo Koyasu, Richard M. Locksley, Andrew N.J. McKenzie, Reina E. Mebius, Fiona Powrie & Eric Vivier.

Nature Reviews Immunology 13, 145-149 (February 2013) | doi:10.1038/nri3365

With respect to the timeline of ILC discovery, the first ILC to be described were a subset of ILC1. In 1975, Kiessling and colleagues described a lymphocyte population poised to “naturally kill” tumors in vitro without any requirement for prior antigenic stimulation¹. Some features of these cells, also known as Natural Killer (NK), were used as a basis upon the discovery of other ILC subsets. In 1992, the identification of unknown lymphocytes that resided in neo-natal lymph nodes further led to their characterization as Lymphoid Tissue inducer cells (LTi)², which are the prototypical member of ILC3. ILC2 were described 20 years later, and were first named “natural helper”³ as opposed to Natural Killer, but also “nuocytes”⁴. As each group named overlapping population differently, an uniform nomenclature was proposed in 2013⁵ (Fig.1). Since then, the number of reports on “innate lymphoid cells” kept increasing bringing more and more insight as to their function and diversity (Graph.1). Due to this dynamic, group investigating ILC subsets had to constantly adjust by adding markers to distinguish subsets that were thought to be homogenous population. As such, I propose to use an informal nomenclature that aims to reduce confusion while being as accurate as possible.



Graph 1 : Increase in Scientific Publications on ILC

**The presented work focuses on ILC1 and ILC3
biology in C57BL/6 mice.**

	Marker	Ligand	ILC1	ILC2	ILC3
	CD45	NA	++	++	+
	CD90	NA	+/***	++	++
	Sca-1	NA	+*	+	+*
Lineage Markers	CD3 (T Cells)	NA	-		
	CD19 (B Cells)	NA			
Transcription Factor	RORγt	NA	+***	-	+
	GATA-3	NA	+	++	+
	T-bet	NA	+	-	+*
	Eomes	NA	+*	-	-
Cytokine Responsiveness	IL-17RB	IL-25	-	+	-
	T1-ST2	IL-33	-	+	-
	CD212	IL-12; IL-23R	+	+**	+
	IL-18R	IL-18	+	-	NA
	IL-23R	IL-23	-	-	+
	IL-1R	IL1α; IL-1β	NA	NA	+
	CD122	IL-2; IL-15	+	NA	NA
	CD127	IL-7; TSLP	+*	+	+
	CD117	SCF	+*	+*	+
Steering Molecules	CCR6	CCL20	-	-	+*
	CXCR6	CXCL16	+*	-	+
	CXCR3	NA	+*	NA	+*
	CD49a	NA	+*	-	+*
	CD49b	NA	+*	-	-
	TRAIL	DR5	+*	-	-
	CD4	NA	-	-	+*
	NKp46	Unknown	+	-	+*
	NK1.1	NA	+	-	-
	KLRG1	NA	+*	+*	-
	MHC-II	TCR	+**	+*	+*

* Subset related
** Following Activation
*** Plasticity
NA Not Adressed

ILC1 regroups three main subsets of NK1.1⁺ NKp46⁺ cells (Fig.2), but no consensus for the nomenclature of these cells has been formally established yet. Out of commodity, I therefore propose a designation based on the expression of the transcription factor Eomes and the integrins CD49a and CD49b that will be used onwards. The features that justify this classification will be addressed in the ILC1 section. I will refer to Eomes⁻ CD49a⁺ ILC1 that resides in the liver and the intestinal mucosa as ILC1^a, to Eomes⁺ CD49b⁺ ILC1, which are commonly known as circulating cytotoxic NK cells as ILC1^b, and to Eomes⁺ CD49a⁺ CD49b⁺ ILC1 that populates the Salivary Gland and the Uterine decidua as ILC1^{ab}. In the case in which the ILC1 subset is not clearly determined (ND), I will refer to as ILC1ND.

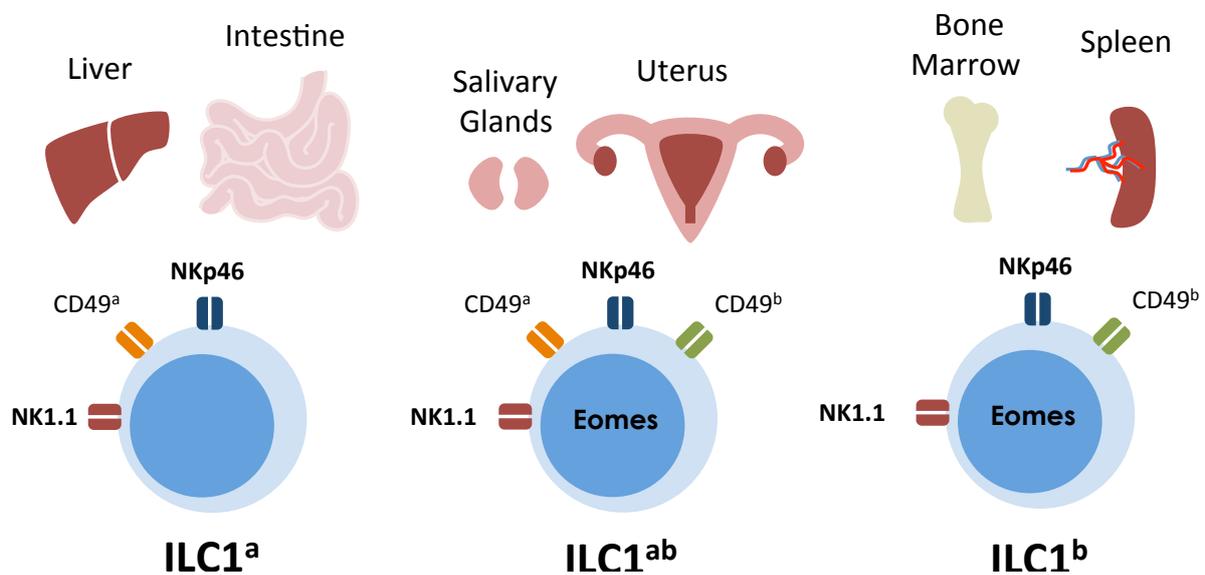


Figure 2 : Group 1 ILC encompass three major populations

ILC3 contains two lineages with a distinguishable ontogeny and different dependency on transcription factors (Fig.3) , which will be further explained in the ILC3 section. Fetal CCR6⁺ T-bet⁻ ILC3 Lti-like cells can be dissected according to CD4 expression, and will be referred to as CD4⁻ or CD4⁺ ILC3. CCR6⁻ T-bet⁺ ILC3 that arise post-natally contain NKp46⁻ and NKp46⁺ ILC3, and will be referred to as NKp46⁻ or NKp46⁺ ILC3. As CCR6 and T-bet expression by ILC3 were reported after the discovery of ILC3, some studies solely relied on CD4 and NKp46 expression. In this case, CD4⁻ NKp46⁻ ILC3, which include CCR6⁺ and T-bet⁺ will be referred to as DN ILC3. If the ILC3 subset is not defined, it will be referred to as ILC3ND.

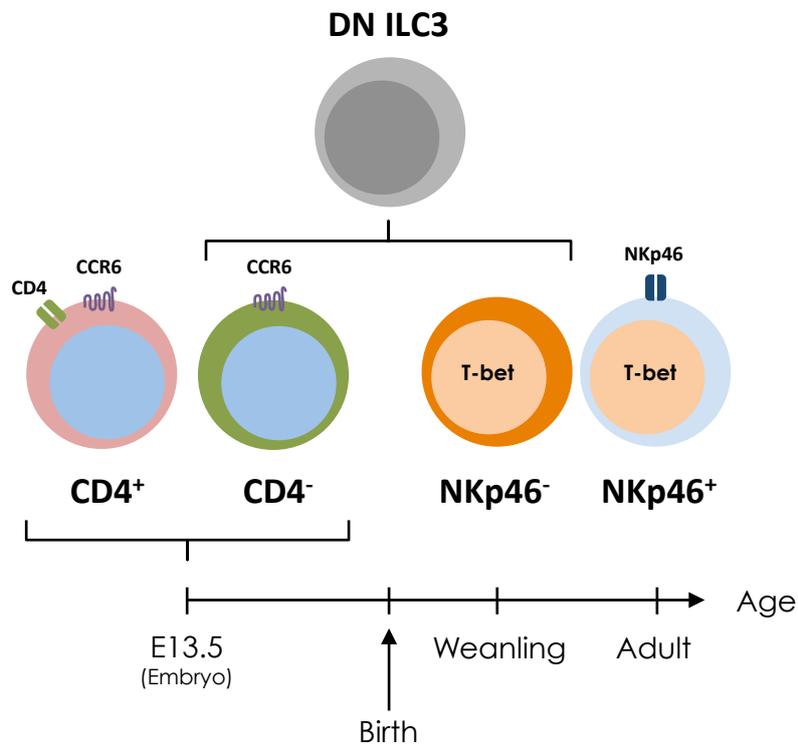


Figure 3 : Group 3 ILC include fetal CCR6⁺ Lti-like cells and T-bet dependent ILC3

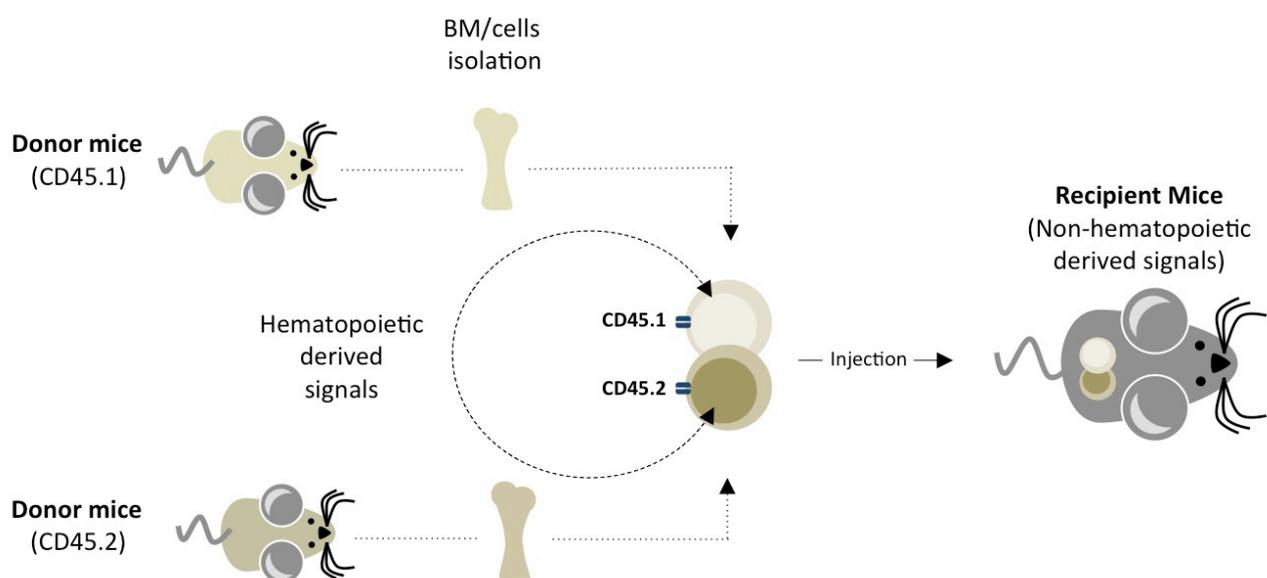
Dissecting Hematopoiesis

Hematopoietic stem cells mature into effector cells through the sequential integration of external signals. Several methods permit to dissect the molecular actors (i.e. cytokines or transcription factors) involved in this differentiation process, and to understand the hierarchy between immune populations.

In vitro culture and adoptive transfer both require isolation of cells, which are either cultured in media with selected cytokines, or injected into recipient mice, respectively. While the signals that drive immune cells development are controlled to some extent *in vitro*, adoptive transfer provides a more complex environment that resembles physiological conditions. If primary cells from genetically modified mice are used as a cellular source, these methods are now crucial to demonstrate both the responsiveness of immune cells to extrinsic signals and their ability to develop in the context of an altered genotype.

As gene ablation in knock-out (KO) mice affects all cells, the phenotype observed in a cell population could be caused by a defect in bystander cells. In order to address whether a gene is involved in an intrinsic fashion for the development or function of a cell population, some of the studies that are presented in this manuscript relied on bone marrow chimera or conditional gene targeting.

BM chimera are generated by injection of a mixture of BM cells isolated from congenically marked mice (i.e. CD45.1 and CD45.2) with a distinct genotype (i.e. WT vs KO) (Fig.4). Hematopoietic cells originating from each donor mice will differentiate into effector cells according to their genotype. If the invalidated gene is required in an intrinsic fashion for a defined population, the phenotype observed in whole KO mice will be recapitulated in BM chimera mice. However, if the phenotype observed in BM chimera mice is similar to WT mice, the signal produced from immune cells derived from the WT compartment compensate for the loss of signals from the KO compartment, indicating



that the defect caused by gene invalidation affects that function of a bystander population. By generating BM chimera with KO mice as recipient, one could distinguish if the effect of gene invalidation is due to an intrinsic or extrinsic defect in hematopoietic cells, and evaluate the contribution of non-hematopoietic cells (i.e. recipient mice) for the function or development of the population of interest. As any cell could exert a bystander effect, the construction of mice that permit targeted gene invalidation based on the « Cre-lox » system facilitated the investigation of intrinsic gene requirement.

Cre-recombinase directly binds and recognizes Lox DNA sequences, which lead to the irreversible excision of the genetic fragment that is flanked by the Lox sequences. Transgenic mice that express Cre under a specific promoter (i.e. lineage specific surface marker or transcription factor), when crossed to transgenic mice bearing Lox sequences, allows for conditional gene targeting (Fig.5) . If Lox sequences flank a coding gene, Cre expression leads to the excision of the gene, thereby invalidating the gene in cells that activated the Cre-controlling promoter. As genetic recombination is transmissible during cellular division, daughter cells will inherit the invalidated gene.

This feature has proved itself crucial for the establishment of « fate-mapping » studies, in which the targeted genetic sequence is often a stop codon that prevents the expression of a reporter protein. Cre mediated recombination leads to the expression of the reporter protein (i.e. fluorescent protein), thereby genetically marking cells in a stable fashion, irrespectively of the transcriptional status of the Cre-controlling promoter, which supposedly reflects the expression of the gene of interest. By assessing whether cells marked with the reporter still express the gene of interest, one can assess the hierarchy between cell populations. If cells express both the gene of interest and the reporter, this reflects expression stability. However, if marked cells stopped expressing the gene of interest, and because Cre expression is mandatory for the labelling of these cells, this indicates that expression of the gene of interest had been lost.

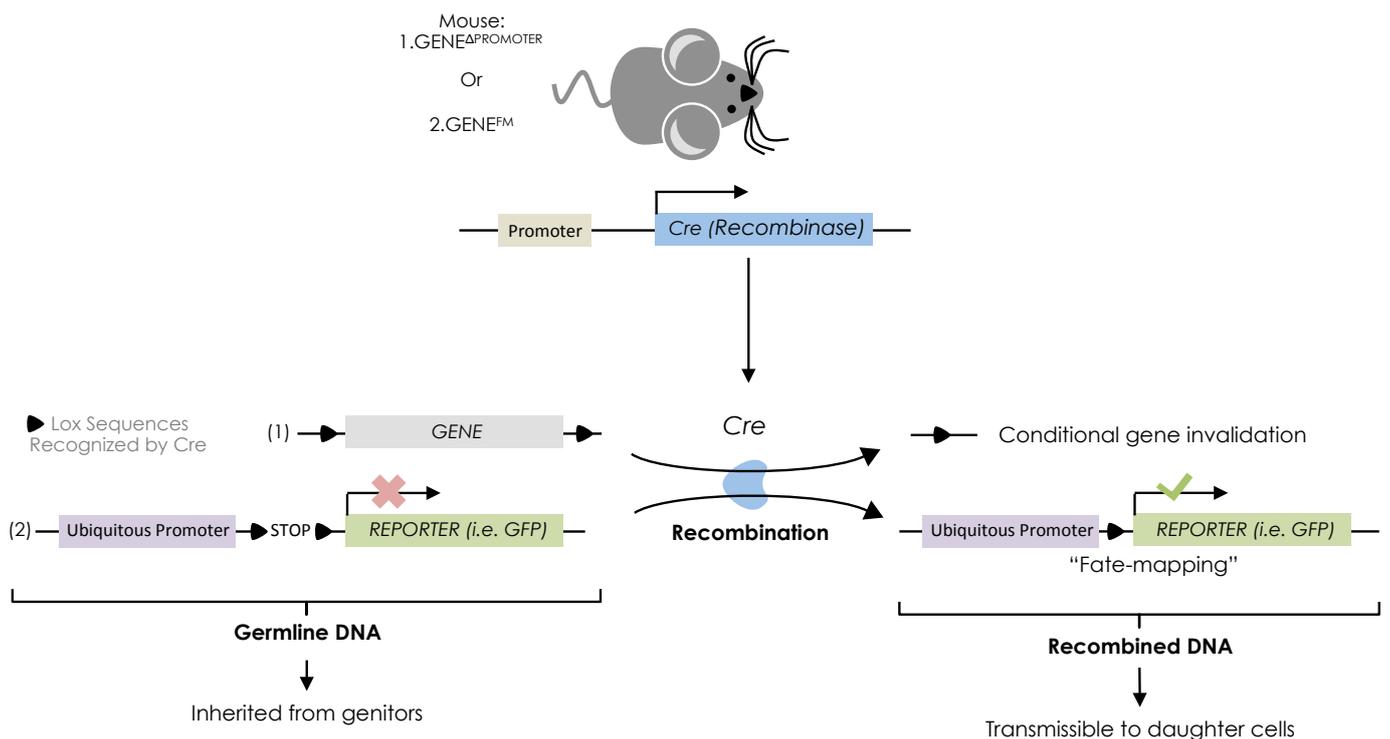


Figure 5 : Cre-Lox mice allow for conditional gene targeting and fate-mapping 17

Paving the way for an ILC fate

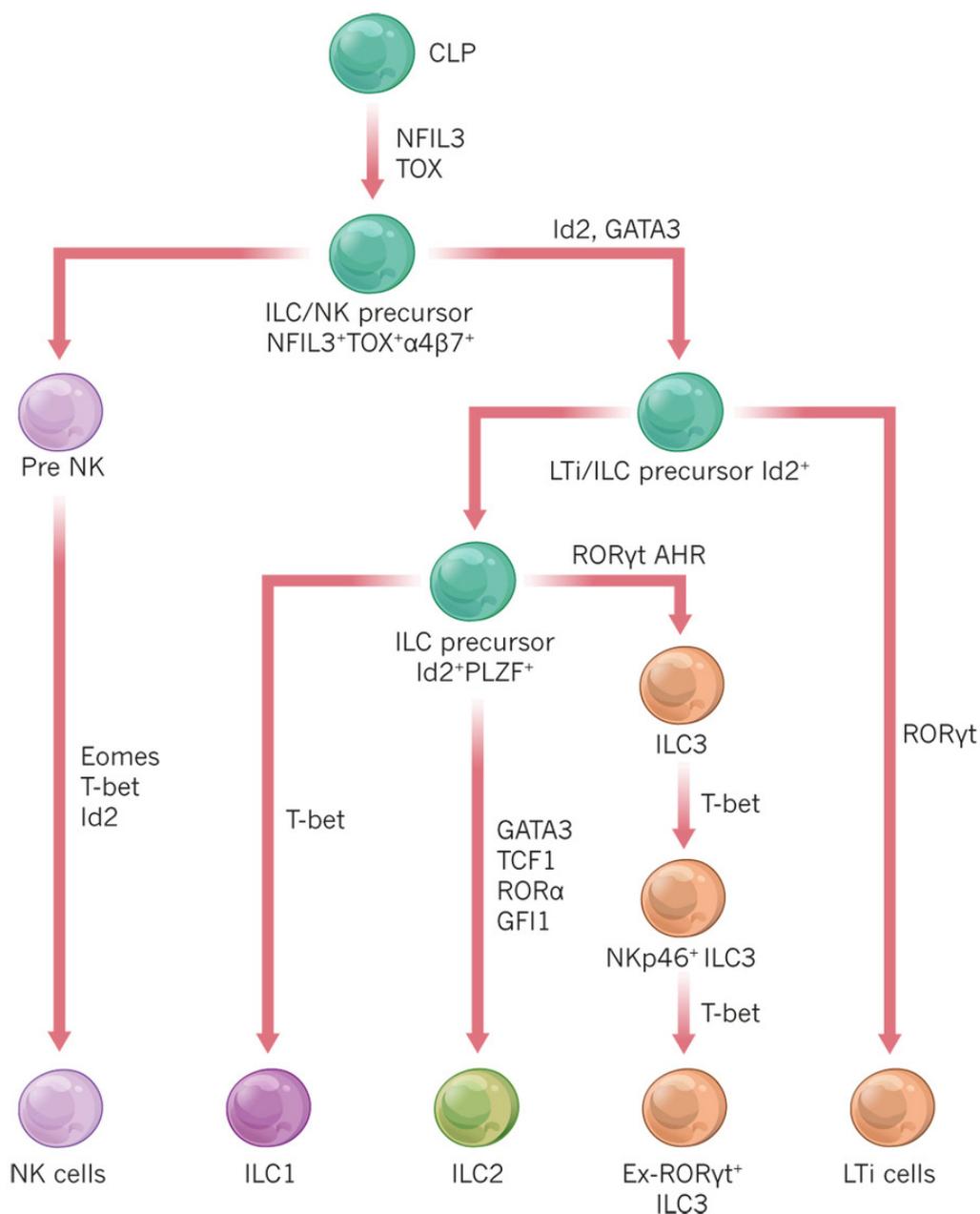


Figure 6 : Model for ILC development (2015)
“The biology of innate lymphoid cells”

David Artis & Hergen Spits
 Nature 517, 293–301 (15 January 2015)
 doi:10.1038/nature14189

Around the 11th day of embryonic life (E11.5), murine HSC begin to colonize the fetal liver (FL)⁶, an environment that fosters their self-renewal and onset their differentiation into hematopoietic lineage. At E17.5, HSC migrate from the liver to the bone marrow⁷ (BM) where they remain throughout life. In both organs, HSC eventually give rise to common lymphoid progenitors (CLP)^{8,9}, which can differentiate into T and B and cells, the two arms of adaptive immunity (Fig.7). CLPs were identified as cells negative for lineage surface markers (Lin^-) bearing surface expression of the IL-7R α chain (CD127 $^+$), the Stem-Cell Factor (SCF) receptor c-kit (CD117 $^+$), the Stem cell antigen 1 (Sca-1 $^+$) and Flt3 (Fms-Like tyrosine kinase 3 or CD135 $^+$)¹⁰. As discussed below, CLPs also retain the potential to generate ILCs, and were used as a basis to identify an ILC-restricted progenitor. ILC function and development share common features with those of T^H cells. Most of the molecules that will be mentioned tend to be better characterized in T^H cells, I will often introduce their role in T^H cells before focusing on ILC.

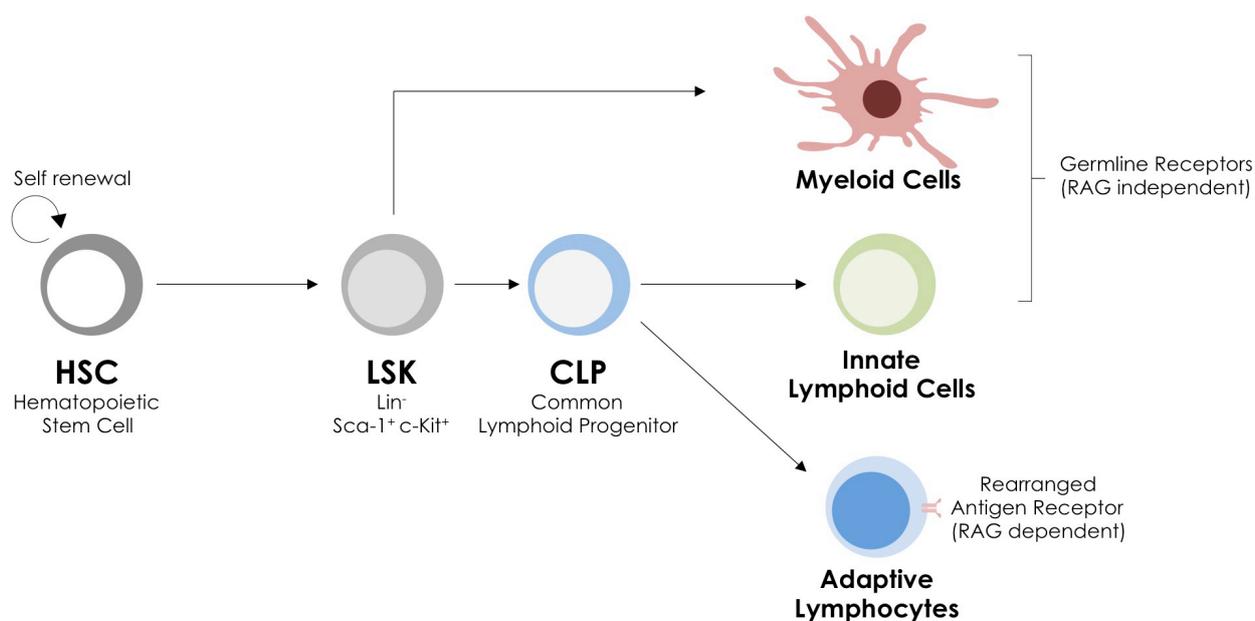


Figure 7 : CLP give rise to RAG-independent ILC and RAG-dependent adaptive lymphocytes

TCF-1 : EILP^{ing} ILC

The Early ILC progenitor (EILP), was characterized according to the expression of the transcription factor T cell factor 1 (TCF-1)¹¹. Encoded by the gene *Tcf7*, TCF-1 acts at various stages in T cell biology.

T cell development occurs in the thymus, where thymocytes, the progenitors that mature into T cells, follow a well-defined pathway (for review, see Shah & Zuniga-Pflücker 2014¹²). Briefly, CD4⁻ CD8⁻, or double negative (DN) thymocytes begin to somatically rearrange their T-cell receptor genes in a RAG dependent manner. DN cells that successfully rearranged the T cell receptor (TCR) gene receive TCR-dependent survival signals, and acquire CD4 and CD8 expression. These Double Positive (DP) thymocytes subsequently acquire CD3 surface expression and a fully mature TCR. They concomitantly lose either CD4 or CD8 to become single positive (SP), therefore specifying either into CD3⁺ CD8⁺ cytotoxic T cells that specialize in the destruction of infected or transformed cells, or CD3⁺ CD4⁺ T^H cells, which regulate other cellular partners during immune responses.

In thymocytes, TCF-1 cooperates with β -catenin to promote the transition from DN stage to DP stage¹³ (Fig.9), while in mature T^H cells, binds the promoter of GATA3 and represses IFN- γ production¹⁴, thereby inducing T^H2 cells development by inhibiting T^H1 cells-associated function.

With respect to ILC biology, TCF-1 deficiency dramatically affects the generation of ILC2 and NKp46⁺ ILC3^{11, 15, 16} and to a lesser extent, ILC1^a and ILC1^b (Fig.10). EILP injected into lymphocyte deficient host (RAG^{KO/KO} *Il2rg*^{KO/KO}) further developed into all ILC subsets (Fig.8), but no T and B, indicating that EILP only generate ILC¹¹. In mice that express a fluorescent protein under the control of the *Tcf7* promoter (*Tcf7*^{GFP}), EILP were identified as Lin⁻ Sca-1⁺ CD117⁺ GFP⁺, and express the gut-homing associated integrin $\alpha 4\beta 7$. This surface phenotype overlaps with that of LSK (Lin⁻ Sca-1⁺ CD117⁺ $\alpha 4\beta 7$ ⁺)¹⁷, which are precursors downstream of HSC, as they lost self-renewal abilities, but upstream of CLPs, as they generate both lymphoid and non-lymphoid cells, i.e. myeloid cells (Fig.7).

Out of commodity, I will now refer to Lin⁻ Sca-1⁺ CD117⁺ CD127⁺ $\alpha 4\beta 7$ ⁺ cells as KAS7 (for c-Kit, $\alpha 4\beta 7$, Sca-1, IL-7 α), which do not represent any identified population but rather reflect the surface markers conserved between the different studies.

EILP lack CD127 expression, and are therefore phenotypically closer to LSK than CLPs. However, whether CLPs generate EILP remain unknown. If EILP are indeed downstream of CLPs, they would represent the “ILC/NK” precursor (Fig.6 versus 8). If CLPs fail to generate EILP, this would indicate the ILC commitment is marked before CD127 expression.

Intriguingly, DP thymocytes from transgenic mice in which β -catenin is rendered resistant to ubiquitin-mediated degradation and subsequently more active, express higher amount of CD127¹⁸, indicating that TCF-1 may require other molecular partner to maintain or trigger CD127 expression in EILP.

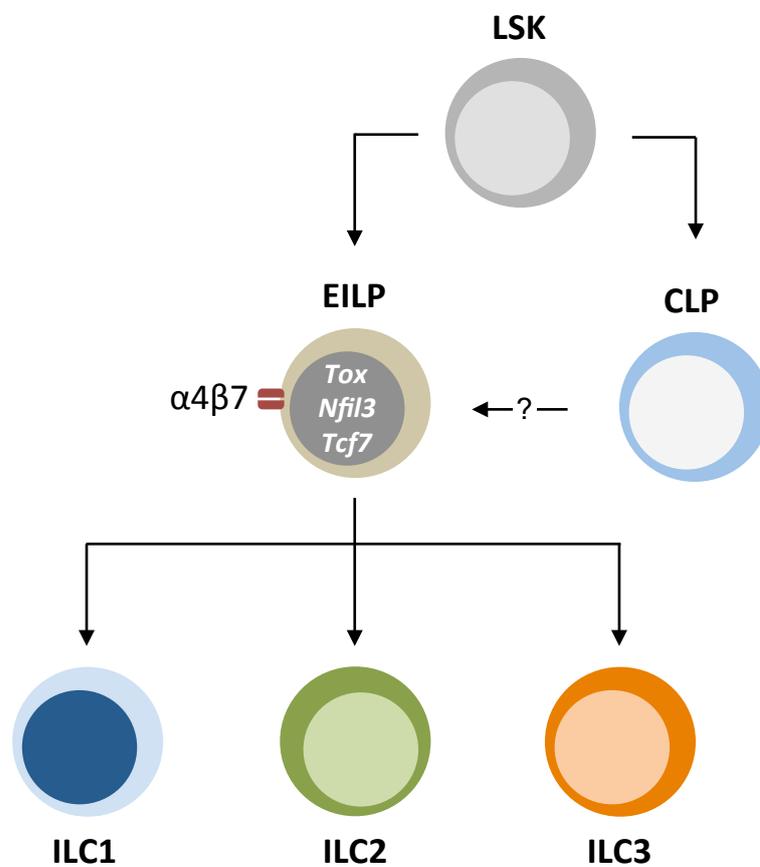


Figure 8 : EILP give rise to all ILC subsets

Nfil3 around the clock

Nuclear Factor, Interleukin 3 regulated (Nfil3) expression is readily detected in EILP¹⁶ (Fig.10). Also known as E4BP4¹⁹ (E4 promoter Binding protein 4), this basic leucine zipper transcription factor is encoded by the *Nfil3* gene, and operates both in hematopoietic and non-hematopoietic cells. For instance, hypothalamic neurons contain mRNA transcripts for *Nfil3*²⁰. The hypothalamus regulates sleep, food intake and hormone production, all of which are largely influenced by environmental cues such as exposure or absence of daylight and temperature. To define this internal clock, termed circadian rhythm, a set of genes, including *Nfil3*, cyclically modulates their expression^{21,22}.

In the hematopoietic compartment, cyclic expression of Nfil3 was reported to suppress T^H17 cells development through direct binding to the *Rorc* promoter, thereby linking circadian rhythm and susceptibility to intestinal inflammation²³. *Nfil3* also directly controls cytokine expression, as it was originally identified through its ability to activate the *Il3* promoter in human T cells¹⁹. In addition, overexpression of Nfil3 drives the production of the T^H2 associated-cytokine IL-13 and the anti-inflammatory cytokine IL-10 by mature T^H1 cells²⁴, reflecting its role in regulating lineage-associated T^H cell function. Nfil3 is upregulated following chronic TCR stimulation of T^H1 cells²⁴, or upon IL-3 stimulation of immature B Cells to promote their survival²⁵.

Regarding the development of dendritic cells (DCs), which belong to the myeloid lineage, the original study by Kashidawa report the absence of a subset of CD8 α expressing DCs in the spleen in *Nfil3*^{KO/KO} mice²⁶. In another report, *Nfil3* deficiency did not impact the generation of CD8 α ⁺ DCs in mice reconstituted with a mixture of BM from wild-type (WT) mice and *Nfil3* deficient mice²⁷. These BM chimera stresses the cell extrinsic role for Nfil3 in the generation of CD8 α ⁺ DCs, which therefore rely on another Nfil3-dependent population for their development.

Remarkably, numerous reports testify that ILC intrinsically depend on Nfil3²⁸⁻³⁶. In our hands, *Nfil3*^{KO/KO} mice are devoid ILC1^a and ILC1^{ab}, which contradicts published report identifying Nfil3-independent ILC1^{ab(32, 34)} and ILC1^{a(37)}, an issue that will hopefully be clarified. Nonetheless, transfer of BM LSK from *Nfil3* deficient mice failed to generate the ILC compartment²⁹, demonstrating that *Nfil3* expression is intrinsically required for ILC development. Conditional deletion of Nfil3 in mature ILC1^{b(30)} or NKp46⁺ ILC3³¹ had no effect on their generation, indicating that Nfil3 act at the early stages of ILC development and is dispensable for their homeostasis.

Interestingly, IL-7 stimulated CLP upregulated *Nfil3* expression *in vitro*, which has also been reported in EILP that lack CD127. These conflicting results imply two outcomes. Either EILP are an intermediate stage between CLPs and mature ILC, and transiently modulate CD127 expression, therefore explaining the presence of *Nfil3*

“Tox Tox Tox”

Encoded by the *Tox* gene, Thymocyte selection-associated HMG box protein (TOX) is a transcription factor originally identified as a gene associated with thymocytes development³⁸.

In DN thymocytes, TOX expression is TCR dependent. Indeed, RAG deficient thymocytes, which fail to rearrange their TCR and are subsequently blocked at an early DN stage, lack TOX expression³⁸. Still, *Tox*^{KO/KO} mice exhibit no defect in DP generation, indicating that although TOX expression is initiated early (Fig.9), it is not required for the DN to DP transition³⁹. Stimulation of DP thymocytes with phorbol 12-myristate 13-acetate (PMA) and ionomycin, an ionophore that triggers Ca²⁺ influx used here to mimic TCR signaling, triggered the upregulation of TOX³⁸.

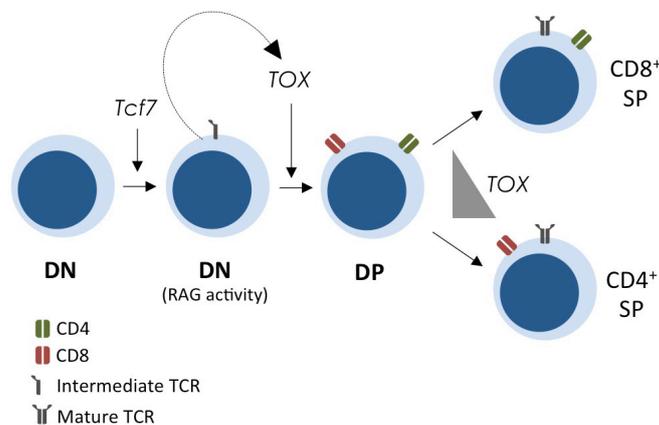


Figure 9 : Tox and Tcf-1 in thymocytes development

However, *Tox*^{KO/KO} lack CD4⁺ SP thymocytes and mature T^H cells, but display a normal CD8⁺ T cell compartment, indicating that *Tox* expression is required for T^H cells commitment, and its absence lead to the generation of CD8⁺ T cells³⁹. Remarkably, transgenic mice, in which TOX expression is driven by the Lymphocyte-specific protein tyrosine kinase (*lck*) promoter, display biased generation toward CD8⁺ SP thymocytes, although these cells failed to egress from the thymus and become mature⁴⁰. These results indicate that the level of intracellular TOX instructs CD4 versus CD8 cell fate decisions in the thymus (Fig.9).

Remarkably, mature ILC also express TOX, which expression was mandatory for their generation. Indeed, pulmonary ILC1^b and ILC2, splenic ILC1^b and ILC3, intestinal ILC1^a, ILC1^b, ILC2 were all affected by TOX deficiency⁴¹. Analysis of TOX/WT BM chimera revealed that this defect was cell intrinsic, as most of the ILC generated derived from the WT compartment.

Reminiscent of thymocytes development, Tox transcripts are detected early in the ILC lineage, as EILP already express TOX¹¹ (Fig. 8). In addition, retroviral transduction of *Nfil3* deficient CLPs with a vector expressing TOX partially rescued the generation of ILC, indicating that TOX acts downstream of *Nfil3* in ILC development³⁶ (Fig. 10). Because ILC do not rely on TCR expression for their generation, the question of whether Tox expression is also controlled by Ca²⁺ influx or other factors such as *Nfil3* will hopefully be answered in future studies.

ID2 : CHILP vous plaît

Proteins of the Inhibitor of DNA Binding (Id) family encompass four members: Id1, Id2, Id3 and Id4. Id proteins are a peculiar form of transcription regulator as they are unable to directly bind DNA. As their name points out, Id proteins rather act by preventing the DNA binding of the E-protein family of transcription factors (for reviews see Kee 2009)⁴². E2A, a member of the E-protein family, is required for the generation of adaptive lymphocytes⁴³⁻⁴⁵. Briefly, E2A deficient mice fail to rearrange B-cell receptor (BCR) and subsequently lack B cells⁴³, while thymocytes exhibit a partial block at the DN stage⁴⁵. The residual T cells that are generated in E2A deficient mice are highly susceptible to transformation and generate lymphoma in older mice⁴⁵. In cultured fetal liver HSC, E2A deficiency led to the generation of ILC1ND and myeloid cells at the expense of T cells⁴⁶. In immature B cells, invalidation of EBF1, an E2A target, led to the reprogramming into ILC2 and ILC3⁴⁷. Here, in the absence of signals require for an “adaptive fate”, commitment was diverted toward innate lineages.

Consistent with this idea, Id proteins, which antagonize E-proteins, were shown to divert progenitors toward an “innate” fate⁴⁸⁻⁵⁰. Overexpression of *Id1* in cultured LSK led to the extinction of B cell and promoted myeloid generation⁴⁸. With respect to ILC1^b and CD4⁺ ILC3, an early report, which preceded the discovery of other ILC subsets, demonstrated that Id2 was crucial for their development⁵⁰. Retroviral transduction of fetal thymocytes with *Id2* abrogated T cell potential and only generated ILC1^{ND50}. While ILC1^b and CD4⁺ ILC3 are absent in *Id2*^{KO/KO} mice, they developed normally in *Id2*^{KO/KO} E2A^{KO/KO(49)}, pointing out that Id proteins acts by antagonizing E2A.

As a matter of fact, all ILC express and depend on *Id2*^{3,37,49,51-55}. This finding was one of the basis for the current classification of ILC.

« We hypothesize that all ILCs develop from a common precursor that may depend on expression of the transcriptional repressor inhibitor of DNA binding 2 (*ID2*) »

Hergen Spits, David Artis, Marco Colonna, Andreas Diefenbach, James P. Di Santo, Gerard Eberl, Shigeo Koyasu, Richard M. Locksley, Andrew N.J. McKenzie, Reina E. Mebius, Fiona Powrie & Eric Vivier.

Subsequently, *Id2* reporter mice allowed for the identification of an ILC precursor, denominated CHILP (Common Helper-like ILC progenitor)⁵⁶. In *Id2*^{GFP} mice, CHILP were defined as KAS7⁺ CD135⁻ GFP⁺. Transfer of CHILP into RAG^{KO/KO} *Il2rg*^{KO/KO} generated ILC1^a, ILC2, and ILC3, but failed to reconstitute the cytotoxic ILC1^b compartment⁵⁶. Analysis of double *Id2*^{GFP} *Tox*^{Tomato} reporter mice revealed that all Lin⁻ $\alpha 4\beta 7$ ⁺ CD127⁺ coexpressed *Id2* and TOX⁴¹ (Fig.10). As CHILP were absent from *Tox* deficient mice⁴¹, *Id2* expression precede that of TOX. In a similar fashion, *Nfil3* deficient mice also lacked CHILP²⁹. Retroviral transduction of *Nfil3* deficient LSK with *Id2* restored the generation of ILC²⁹. Chromatin immunoprecipitation with an anti-*Nfil3* antibody revealed the enrichment for *Id2* sequences, indicating that *Nfil3* directly bind the *Id2* gene²⁹.

Ergo, CHILP requires *Tox* and *Nfil3*, and display a more restricted potential than EILP, as they fail to generate cytotoxic ILC1^b. CHILP appears therefore to be downstream of EILP (Fig.10).

PLZF - Same-Same, but different.

NK T cells (NKT) are a family of lymphocyte that, like T^H cells, originate from CD4⁺ SP thymocytes and are RAG dependent (for review see Constantinides & Bendelac 2013⁵⁷). NKT effector functions largely rely on the transcription factor PLZF (Promyeolocytic Leukaemia Zinc Finger, encoded by *Zbtb16*), as PLZF deficient NKT fail to mature and constitutively migrate to lymph nodes and spleen, from where they fail to egress⁵⁸.

Although mature ILC are negative for PLZF⁵⁹, **fate-mapping** analysis revealed that a fraction of them had a prior history of PLZF expression. The majority of ILC2 from the lung and the BM, as well as hepatic ILC1^a and intestinal NKp46⁺ ILC3 originated from PLZF expressing cells⁵⁹. To a lesser extent, CD4⁻ and CD4⁺ ILC3, as well as splenic and hepatic ILC1^b were also “fate-mapped” for PLZF expression. Consequently, BM KAS7 cells contained a fraction of PLZF expressing cells, which will be now referred to as PLZF⁺ ILCP(recursor)⁵⁹. These cells expressed high amount of transcripts for *Tox*, *Id2* and *Tcf7*, features that are reminiscent of CHILP.

In vitro culture of PLZF⁺ ILCP could generate ILC1ND, ILC3ND and ILC2. In *Zbtb16*^{KO}/^{KO}/WT BM chimera ILC2 and hepatic ILC1^a were only derived from WT donor, indicating that, although PLZF expression marked lymphoid progenitors with ILC potential, it was only required for ILC2 and hepatic ILC1^a development. Adoptive transfer of PLZF⁺ ILCP in RAG^{KO/KO} *Il2rg*^{KO/KO} generated pulmonary ILC1ND and hepatic ILC1^a, ILC2 in the lung and the small intestine, and ILC3ND, but no T and B cells. As a matter of fact, CHILP contained a fraction of PLZF⁺ and PLZF⁻ cells⁵⁶. PLZF⁺ ILCP numbers were also decreased in mice deficient for *Nfil3*²⁹ and *Tcf7*⁴¹, and therefore appear to be downstream of EILP, and closely related to CHILP (Fig.10).

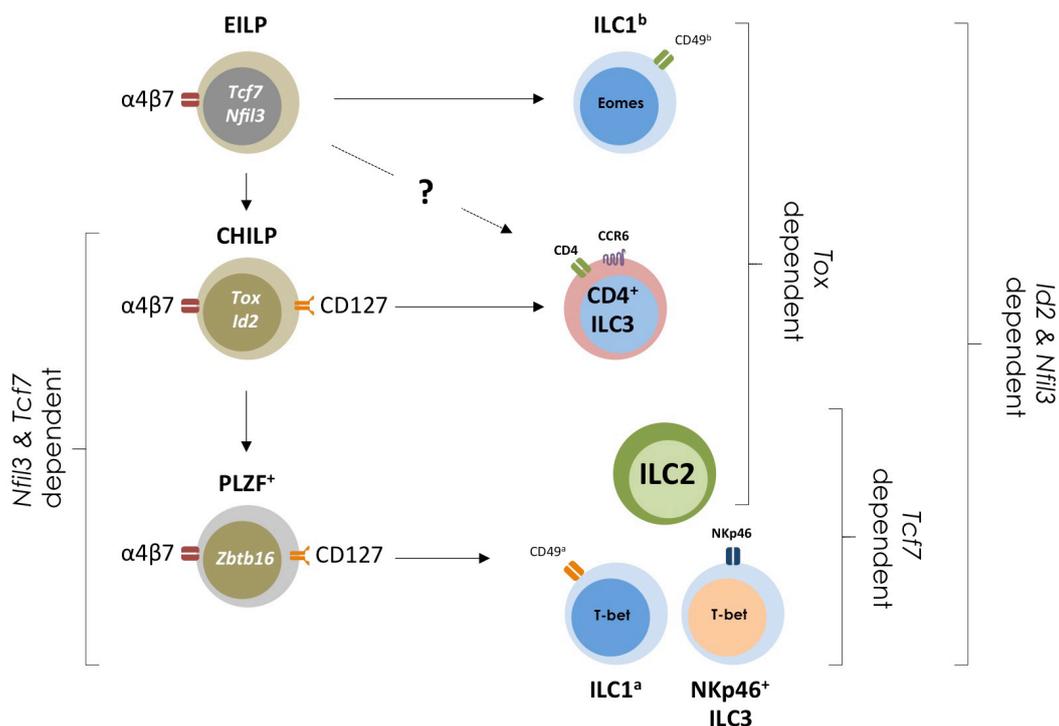


Figure 10 : Recapitulating Scheme of ILC commitment

COMMON GAMMA CHAIN : One to rule them all

Patients with Severe ImmunoDeficiency Syndrome (SCID) lack viable adaptive immunity and are sometimes forced to live in sterile environment (for review see Fischer et al. 2015)⁶⁰. Several invalidating mutations cause SCID, including the ones that affect the *Il2rg* gene. In human and mice, *Il2rg* encodes the common γ chain of the IL-2 receptor, or CD132.

The first characterization of *Il2rg*^{KO/KO} mice revealed that ILC1^b were severely affected by lack of CD132⁶¹. As these mice still possess residual T and B cells, *Il2rg*^{KO/KO} were backcrossed to RAG to generate alymphoid RAG^{KO/KO} *Il2rg*^{KO/KO(62)}. As mentioned in the above sections, RAG^{KO/KO} *Il2rg*^{KO/KO} mice are commonly used as hosts for transfer experiments. Indeed, organs of RAG^{KO/KO} *Il2rg*^{KO/KO} mice are devoid of lymphocytes, and therefore provide an empty niche that can eventually be filled up by injected cells.

Later, RAG^{KO/KO} *Il2rg*^{KO/KO} were shown to be also devoid of ILC2^{3, 56, 63} and ILC3^{64, 65}, such that common γ chain dependency is a common feature of ILC.

Cytokines for which cognate receptors associate with, and therefore require, CD132 form the family of common γ chain cytokine, which includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. As there are extensive reviews that discuss their role in T^H cells (see Rochman et al. 2009, Yamane & Paul. 2012)^{66, 67}, I will first briefly present the distinct dependency of ILC toward IL-7 and IL-15.

Acquisition of CD127 in hematopoietic cells progenitors is associated with an increased potential to generate lymphocytes^{8,9}. Encoded by the *Il7ra* gene, CD127 associates with CD132 to form a functioning receptor that permits IL-7 responsiveness (Fig.11). With respect to ILC precursor, only one report addressed IL-7 requirement *in vivo*, showing that Lin⁻ CD117⁺ α 4 β 7⁺ Flt3⁻ cells were unaffected in *Il7ra* deficient mice⁵⁶. Because CD127 could not be used as a discriminating marker in these mice, whether these cells represent EILP, or downstream CHILP or PLZF⁺ ILCP, is still unknown. In addition, Thymic Stromal-derived Lymphopoietin (TSLP) signal through TSLPR that requires CD127 to transmit signal⁶⁸ (Fig.11). Still, Mature ILC2 and ILC3 differ from ILC1 by their dependency on CD127, and are virtually absent in *Il7ra*^{KO/KO} mice^{52, 64, 69} in *Il7ra*^{KO/KO(54, 70)}, while ILC1^b numbers (except for a subset that resides in the thymus)⁷¹ are unaffected in *Il7ra*^{KO/KO(56, 70)} or *Il7ra*^{KO/KO} mice^{52, 71}.

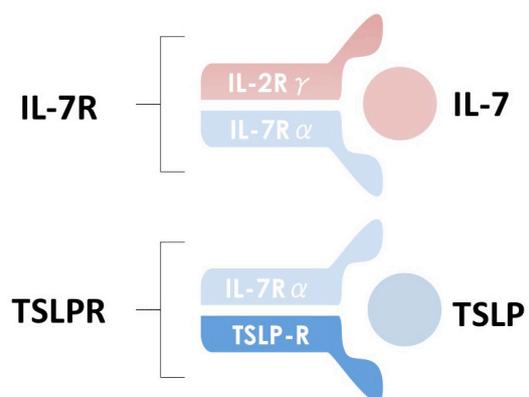


Figure 11 : Schematic rRptors involved in ILC survival

However, mice deficient for IL-15 are devoid of all ILC1^{34, 52, 72, 73}. As a matter of fact, acquisition of CD122 expression is an early event that marks commitment to ILC1^b fate. Indeed, a fraction of Lin⁻ CD122⁺ (referred to as NK cells precursor, or NKp) could generate ILC1^b *in vitro*, and lacked myeloid, T and B cell potential⁷⁴. Analysis of BM Lin⁻ CD127⁺ cells revealed that CD135, which identify CLP, and CD122 expression⁷⁵, were mutually exclusive. Remarkably, all Lin⁻ CD127⁺ CD135⁻, either CD122⁻ (referred to as pre-NKp) or for CD122⁺ (referred to as r(eefined)NKp), generated splenic ILC1b, but no T and B cells upon transfer in RAG^{KO/KO} *Il2rg*^{KO/KO(75)}.

Regarding the transcription factors involved in ILC commitment, *Nfil3* expression is detected in BM preNKp, and increases from rNKp to ILC1^{b(29, 76)}. As *Tcf7*^{ko/ko} mice lack NKp⁴¹, they can be considered downstream of EILP. Finally, CHILP are described as Id2⁺ α4β7⁺ CD122⁻ that can generate all ILC except for ILC1^{b(56)}. Although NKp are Id2 independent⁴⁹, a fraction of NKp expresses Id2⁷⁷, and generated more ILC1^b than their Id2⁻ counterpart *in vitro*, implying a role for *Id2* in later stage of ILC1^b development,.

Overall, CD122 and CD127 expression by ILC progenitor, and therefore IL-7 and IL-15 responsiveness, appear to be important in the branching of ILC1^b versus other ILC subsets (Fig.13).

I GATA3 my mind

GATA3 belongs to the GATA family of transcriptions factors, which binds conserved WGATAR (W for A or T, and R for A or G) motif⁷⁸. GATA3 deficient embryos are non viable and die around E11.5⁷⁹. Therefore, most of the studies that investigated GATA3 function in the immune system in vivo either relied on hematopoietic FL or conditional invalidation.

Briefly, GATA3 is required for T-cell development at several stages. Conditional invalidation of GATA3 driven by the *lck* promoter leads to a huge decrease in DP thymocytes, which reflects its role in the DN to DP transition, and driven by the *Cd4* promoter, which expression is onset at the DP stage, lead to a marked decrease in CD4 SP⁸⁰. Although CD8 SP numbers are unaffected, CD8⁺ T cells further require GATA3 to exhibit normal cytotoxic function driven by TCR signaling, as well as normal proliferation⁸¹. Overall, GATA3 participates in the CD4/CD8 lineage determination, and in the effector functions of mature CD8⁺T cells (Fig.11). GATA3 also hallmarks T^H2 responses, as it controls the expression and binds the promoters of *Il4* and *Il13*^{82, 83}.

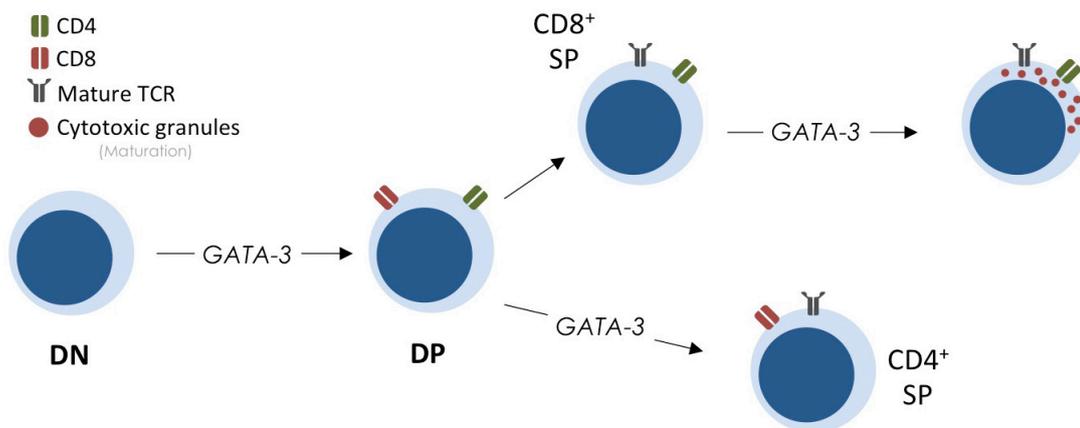


Figure 11 : GATA-3 in thymocytes development

GATA3 appears to instruct ILC in a similar way to T-cells. The generation of ILC1^a, ILC2 and ILC3 was totally abrogated following transfer of GATA3 deficient FL cells or in mice with conditional deletion in the hematopoietic compartment^{84, 85}. The defect was observed at an early stage, as Lin⁻ CD127⁺, which includes ILC precursors, were all absent in the FL in both models. GATA3 is therefore required for the development of non-ILC1^b ILC subsets. However, maturation of ILC1^b was markedly affected, both upon adoptive transfer of GATA3 deficient FL cells or in mice that conditionally lacked GATA3 in the ILC1 compartment^{86, 87}. Both studies demonstrated that GATA3 deficiency reduced the number CD11b⁺ KLRG1⁺ ILC1^b, which marks the final stage of maturation,

and a defect in IFN γ production. In addition, GATA3 deficiency upregulated the chemokine receptor CXCR4 expression by BM ILC1ND, known to be involved in the retention of ILC1ND in the BM⁸⁸. Blockade of CXCR4 with a chemical inhibitor allowed ILC1ND to efficiently egress from the BM.

In *Gata3*^{GFP} mice, a fraction of LSK are GFP^{+54,89}, while *Gata3* transcripts are reduced in *Nfil3* deficient Lin⁻ BM cells²⁸, but present in PLZF⁺ ILCp⁵⁹. Stainings for GATA3 protein in CHILP or NKp were almost indistinguishable from the negative control^{56,87}, and the report identifying EILP did not address GATA3 expression. Still, analysis of ILC3 from mice in which GATA3 is conditionally deleted upon administration of doxycycline, revealed that *Id2* and *Tcf-7* were not affected by *Gata3* deficiency, possibly excluding a role for GATA3 in initiating *Id2* and *Tcf7* expression in precursor⁸⁵. As such, *Gata3* expression appears to be initiated after *Nfil3* and *Tcf7*, and is presumably downstream *Id2* and upstream PLZF.

T-BET or not T-BET

T-bet belongs to the T-box family of transcription factors, which play diverse role during embryonic development (for review, see Papaioannou 2014)⁹⁰. Encoded by the gene *Tbx21*, T-bet directs T^{H1} responses (for review, see Lazarevic et al. 2013)⁹¹. However, unlike GATA3⁹², T-bet is absent in mature T-cells from the spleen⁹³, and is first induced by IFN γ ⁹⁴ (Fig.12).

Upon activation, TCR signaling first blocks IL-12R β 2 (encoded by *Il12rb2*) expression therefore inhibiting IL-12 responsiveness. This transcriptional block is then relieved when TCR signaling stops, thereby allowing IL-2 mediated *Il12rb2* transcription, which permits IL-12 to induce T-bet expression⁹⁴. *Il12rb2* and *Ifng* are both direct T-bet target genes, and signaling through their cognate receptors also induce T-bet expression, providing two amplifying loops for T^{H1} responses^{95,96}. In addition to *Ifng* and *Il12rb2*, T-bet was shown to be required for the induction of T^{H1} associated genes such as chemokine (*Xcl1*), chemokine receptors (*Cxcr3*, *Ccr5*) and cytokine receptors such as *Il18r1*⁹⁷.

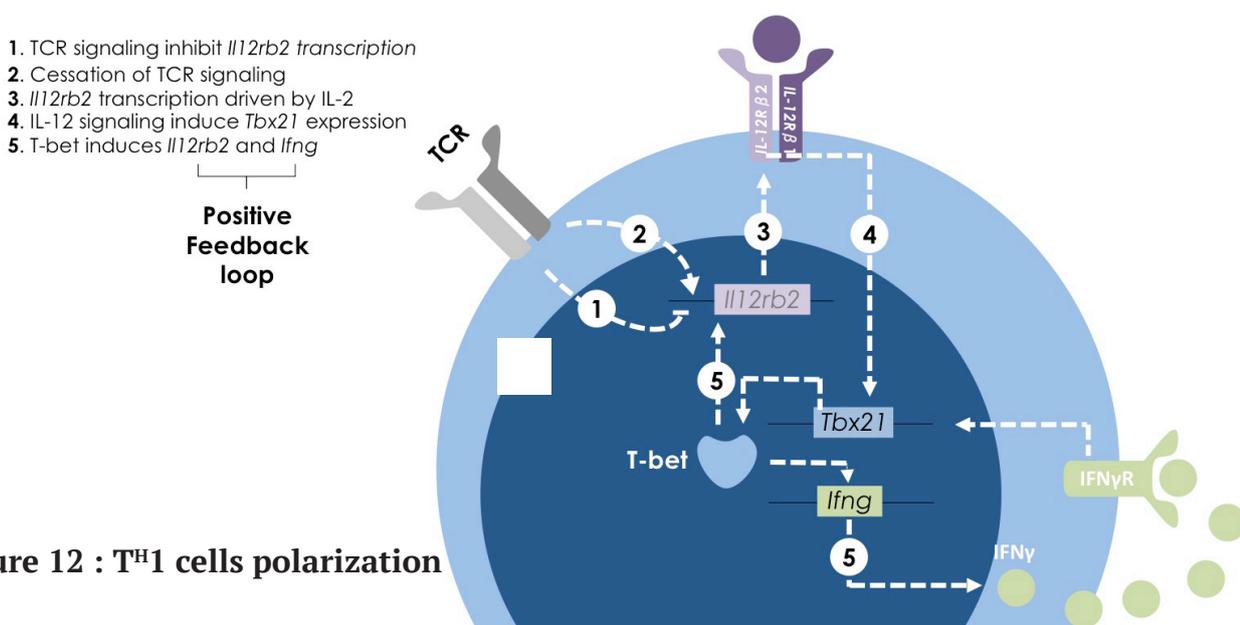


Figure 12 : T^{H1} cells polarization

In the early phase of TCR signaling, T-bet and GATA3 antagonize each other to determine Th1 versus Th2 polarization, as a phosphorylated form of T-bet directly interact with GATA3 and subsequently prevents T^H2 gene transcription⁹⁸. *Tbx21* deficient CD8⁺ T cells fail to acquire normal effector function upon antigen stimulation, display reduced IFN γ production and cytotoxic potential, and fail to elicit recall response in case of a second antigen encounter in vivo⁹⁹. Overall, T-bet antagonize T^H2 fate and function, and promote T^H1 responses.

As such, all ILC1 are T-bet⁺, although they display distinct dependency on *Tbx21* expression. *Tbx21* deficient mice completely lack intestinal and hepatic ILC1^{a(56, 100, 101)}, while SG ILC1^{ab} appear unaffected³². Remarkably, transgenic mice overexpressing T-bet develop more ILC1^a at the expense of ILC1^{b(101)}. However, splenic *Tbx21* deficient ILC1^b exhibit a defect in maturation, increased apoptosis and compensate by proliferating more than their WT counterpart¹⁰². ILC1ND seem to accumulate in the BM of T-bet deficient mice, which is linked to lower expression of *S1pr5*¹⁰³, a T-bet-controlled chemokine receptor involved in the egress of ILC1^b from the BM^{103, 104}, although T-bet expression is not detected in preNKp, nor in proNKp, and unaffected by *Nfil3* deficiency.

Still, as they lack TCR, whether IFN γ or IL-12 is, like in T cells, responsible for the onset of T-bet expression in ILC precursor will probably be addressed in future studies.

EOMESsing around

Although IFN γ expression is controlled by T-bet, it can still be produced in *Tbx21* deficient mice⁹⁹, suggesting that another transcription factor might compensate for the loss of T-bet induced IFN γ expression. Eomes (encoded by *Eomesodermin*) also belongs to the family of T-box proteins. In absence of *Eomesodermin*, embryos fail to develop beyond the blastocyst stage¹⁰⁵.

In the hematopoietic lineage, *Eomesodermin* was originally reported to be expressed by activated or resting cytotoxic CD8⁺ T cells¹⁰⁶. CD8⁺ T cells isolated from Eomes haplodeficient also exhibited reduced amount Granzyme B, a molecule involved in the cytotoxic program¹⁰⁶. The finding that *Tbx21*^{KO/KO} Eomes^{KO/WT}, in addition to their lack of T^H1 cells due to the loss of T-bet, had a marked defect in the cytotoxic lymphocytes splenic compartment, i.e. CD8, NKT and ILC1^b, suggested a role for Eomes in their function¹⁰⁷. Retroviral transduction of CD8⁺ T cells a dominant negative form of Eomes led to reduced CD122 expression. Eomes was then shown to directly bind the *Il2rb* promoter, and subsequently maintained homeostasis of IL-15 responsive cells¹⁰⁷. Remarkably, CD8⁺ T cells that lacked both Eomes and T-bet totally lose the ability to

produce IFN γ , and display aberrant IL-17 production¹⁰⁸, indicating that they are redirected toward the T^H17 lineage. Overall, Eomes appear to control the establishment of the CD8 cytotoxic program while restricting a T^H17 potential.

Conditional invalidation of Eomes in the hematopoietic compartment demonstrated that ILC1^b generation critically relies on this transcription factor, while ILC1^a, which lack Eomes expression, are unaffected¹⁰⁰. *In vitro*, Eomes invalidation in ILC1^b led to the loss of ILC1^b associated marker such as CD49b and the acquisition of ILC1^a marker TRAIL and fail to affect T-bet expression. Eomes ablation in *Tbx21*^{KO/KO} ILC1^b led to the complete loss of ILC1 markers, i.e. NK1.1 and NKp46¹⁰⁰.

Total Lin⁻ CD122⁺, which includes pre-NKp and rNKp, are still present in mice lacking both T-bet and Eomes¹⁰⁰. However, transcripts for *Eomesodermin* are detected in CD122⁻ preNKp and CD122⁺ rNKp⁷⁶, Eomes therefore appear to precede CD122 expression in ILC1^b. In the BM, T-bet and Eomes expression are inversely correlated, at least in ILC1^{b(101)}. Remarkably, retroviral transduction of Eomes in Lin⁻ BM cells isolated from *Nfil3* deficient mice rescued the generation of ILC1^{b(76)}, indicating that Eomes acts downstream of *Nfil3* in ILCp.

While T-bet controls ILC1^a generation, Eomes directs ILC1^b lineage program, and might intervene early in ILCp in the branching between cytotoxic ILC1^b and non-cytotoxic ILC. Interestingly, Eomes has been shown to directly interact with and prevent GATA3 binding to the *Il5* promoter in activated T^H2 cells¹⁰⁹, which may involve an antagonistic functions role of these two transcription factors in cytotoxic/non cytotoxic ILC lineage determination. As such, Eomes appear to be tightly linked to CD122 expression, and its expression should be onset before CD122⁻ CD127⁺ CHILP and PLZF⁺ ILCp.

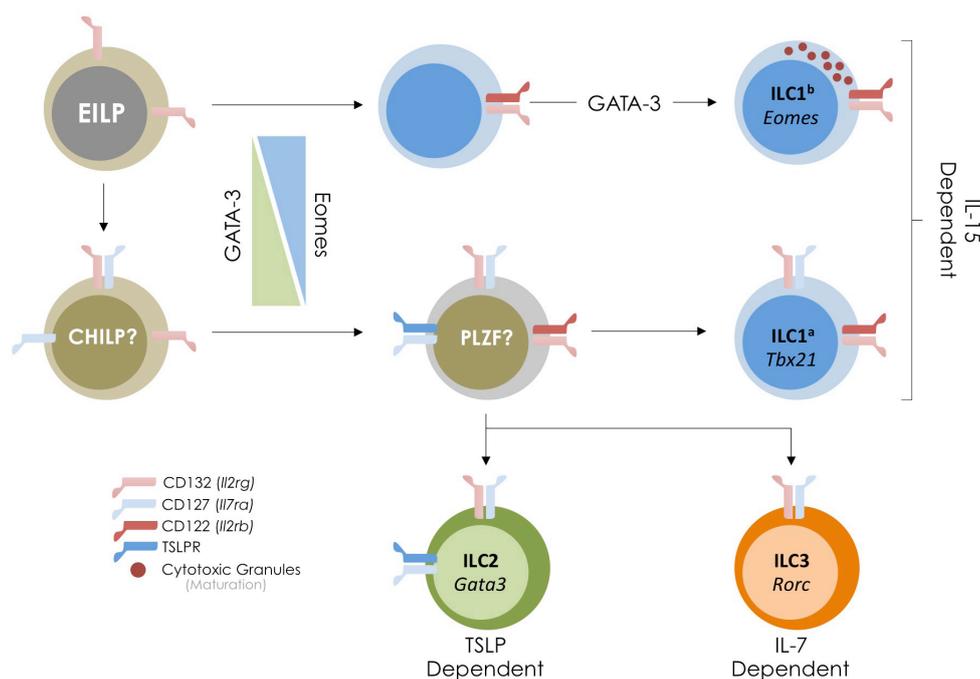


Fig 13 : Cytokine receptor expression in cytotoxic/noncytotoxic fate

ROR γ t – Let him roar again

The Retinoic-acid related Orphan Receptors (ROR) are part of the Nuclear Receptors (NRs) family (for review, see Jetten 2009)¹¹⁰, which regroups transcription factors that modulate their transcriptional activity through the binding of endogenous and/or exogenous ligands. The *Rorc* gene encodes two isoforms of the gamma member of the RORs (ROR γ)^{111, 112} through alternative splicing, among which transcripts for one are highly enriched in the thymus, thereby naming the associated protein ROR γ t^{112, 113}.

ROR γ t expression is readily detected in thymocytes, weaker in RAG^{KO/KO} thymocytes that lacks TCR, yet increases upon *in vivo* treatment of RAG^{KO/KO} with anti-CD3 activating antibody, a method used to mimic TCR signaling, therefore implying TCR signaling for its induction¹¹³. *In vitro* overexpression of ROR γ t reduced the apoptosis of a T-cell line following PMA-ionomycin or anti-CD3 stimulation, pointing out its survival-promoting role¹¹². As such, ROR γ t deficient mice exhibit a relative increase in DN thymocytes, as the residual DP fail to rearrange TCR and subsequently undergo massive apoptosis¹¹⁴. Still, some residual T-cells that escaped apoptosis develop normally and egress the thymus to reach the periphery¹¹⁴. Consequently, transient expression of ROR γ t actively participates to the generation of T cells.

As a matter of fact, sustained ROR γ t expression is mandatory for the generation of pro-inflammatory T^H17 cells^{115, 116}. Overexpression of ROR γ t led to increase production of IL-17¹¹⁵ and upregulation of the IL-23 receptor (*Il23r*) in undifferentiated T^H cells *in vitro*¹¹⁶. However, isolated T^H cells from ROR γ t^{GFP/GFP} deficient mice display a marked decrease in IL-17 production, and fail to upregulate *Il23r* expression¹¹⁵. IL-23 stimulation of polarized T^H17 cells further stabilized ROR γ t expression¹¹⁶, stressing its role in controlling T^H17 functions. As such, ROR γ t drive IL-23 responsiveness, and participate in IL-17 expression, which hallmarks T^H17 responses.

Several compounds were reported to activate ROR γ t transcriptional activity through its ligand-binding domain¹¹⁷⁻¹²⁰. Particularly, lipids that derive from cholesterol metabolism, such as demosterol and zymosterol, which are precursors for cholesterol^{117, 120}, or 7 β ,27-dihydroxy-cholesterol (7 β ,27-OHC)¹¹⁸, a cholesterol product. Remarkably, T^H cells deficient in CYP27A1, the enzyme that generate 7 β ,27-OHC, were less prone to produce IL-17 compared to their WT counterpart¹¹⁸. Here, cholesterol derivatives provided a cell-endogenous source of ROR γ t agonist, linking immune responses to metabolic activity.

Remarkably, Retinoic Acid (RA), a vitamin A metabolite known to instruct development in vertebrates (for review, see Cunningham & Duester 2015)¹²¹, was reported to directly upregulate *Rorc* expression in isolated fetal CD4⁻ and CD4⁺ ILC3¹²². In addition, transgenic mice that expressed a truncated form of the RA receptor display lower ROR γ t expression by fetal CD4⁻ and CD4⁺ ILC3¹²². Whether RA induces ROR γ t in adult ILC3, which include NKp46⁻ and NKp46⁺ ILC3, has not been addressed. Nonetheless, this study still provides important insight as to which stimulus control ROR γ t expression in ILC.

Most importantly, ROR γ t^{GFP/GFP} deficient mice totally lack ILC3^{123, 124}. However, ROR γ t appear to be dispensable in mature ILC3 for their function¹²⁵, and neither ILC1^{123, 124} nor ILC2^{3,4} require ROR γ t for their generation. Fate mapping experiment revealed that, except for a subset of ILC1^{52,70}, neither ILC1 nor ILC2 originated from ROR γ t expressing cells^{52, 126}. With respect to the interaction with other transcription factors, T-bet indirectly prevented ROR γ t-mediated transcriptional activity in T^H cells⁹¹. As EILP, CHILP and PLZF⁺ ILCp are ROR γ t⁺, its expression is probably onset *in situ* in response to external stimuli.

Concluding remarks :

Some studies addressed other features associated with ILC development (for the effect of Notch signaling see Cherrier et al. 2010¹²⁷, for CXCR6⁺ ILCp see Possot et al. 2011¹²⁸, for fetal precursors see Bando et al. 2014¹²⁹, for ILC1 precursor see Constantinides et al. 2015¹³⁰), but replacing them would render the scheme I presented more confusing, and is not necessary for the points I want to address in my discussion. I also chose not to emphasize on factors involved in ILC2 commitment, as this manuscript focuses on ILC1 and ILC3.

Overall, commitment to ILC lineage occurs in a sequential manner. Early expression of *Tcf7* and *Id2* allow for the extinction of B and T cell potential, respectively. Some transcription factors like *Nfil3* may act downstream to stabilize the ILC precursor pool, as their expression is dispensable in mature cells. Strikingly, the development of ILCs branches with ILC1^b cells fate decision, which could be attributed to *Eomes* expression. Surely, the timely combination of transcription factors allows for the guiding to an ILC fate.

Tissue residency : Home Sweet Home

The above section presented evidences that BM hematopoietic precursors sequentially commit to an ILC fate. Intravenous injection of committed ILC precursor into recipient mice generates mature ILCs, indicating that, through the blood circulation, precursors are able to reach tissues where they ultimately differentiate into effectors. Nonetheless, in normal conditions, the pool of mature ILC could be either generated through constant influx of cells from the bone marrow, or maintained by cells that reside in the tissue and proliferate *in situ*.

To discriminate between these two situations, two mice, identifiable through the expression of distinct congenic markers, are surgically “joined” for them to share the same blood circulation. This experiment, called blood chimerism or parabiosis, allows to address whether molecules or cells generated in one mouse are transferable to another through the blood stream, and therefore also used to assess the anatomical provenance of cells inside tissues. If cells originate from the blood circulation, the proportion between cells originating from each mice eventually equilibrate to a 1:1 ratio, indicating that both mice contribute equally to replenish the pool of mature cells in tissues. However, for tissue-resident cells, circulating cells originating from one mouse would fail to replace cells that already reside in the other mouse.

Analysis of parabionts revealed that most ILCs are tissue residents^{72, 131, 132}. Indeed, thirty days post-surgical joining of CD45.1⁺ and CD45.2⁺ mice¹³¹, ILC1^a, ILC1^{ab}, ILC2 and ILC3 were all CD45.1⁺ in CD45.1 mice, and CD45.2⁺ in CD45.2 mice, while blood lymphocytes such as B and T cells were a mixture of CD45.1⁺ and CD45.2⁺. Therefore, with the exception of ILC1^b, which were equally derived from CD45.1⁺ and CD45.2⁺ cells, all the other ILC subsets originated from the host mouse.

This finding is of paramount importance, as tissue-resident cells homeostasis, function and proliferation is therefore driven by tissue-signals received *in situ*, thereby increasing their reactivity and promptness toward harmful stimuli.

Chapter II

Activation & Function – Godspeed you!

As part of the innate immune system, myeloid cells are engaged early during immune responses and, like ILCs, solely rely on germ-line encoded receptors for their generation, homeostasis and function. Sensing of microbial derived-products through Toll Like Receptors (TLR, for reviews see Akira & Takeda 2004, Trinchieri & Sher 2007, O’neill et al. 2013)^{133–135}, most of which require the intracellular Myd88 adaptor, drive the production of cytokine that mediate ILC activation. As extensive reviews summarize the role of these cytokine in pathology and immune activation, I will briefly introduce them before addressing their relative role in instructing ILC1 and ILC3 function. The major cytokines that direct ILC1 and ILC3 responsiveness can be classified into two families, the IL-12 (see Gorieli et al. 2008, Teng et al. 2015)^{136, 137} and the IL-1 (see Dinarello 2009, see Sims & Smith 2010)^{138, 139} family.

In this chapter, I will present some of the ILC1 and ILC3 derived cytokines, and replace the two cytokines families (IL-12 and IL-1) in the context of ILC activation.

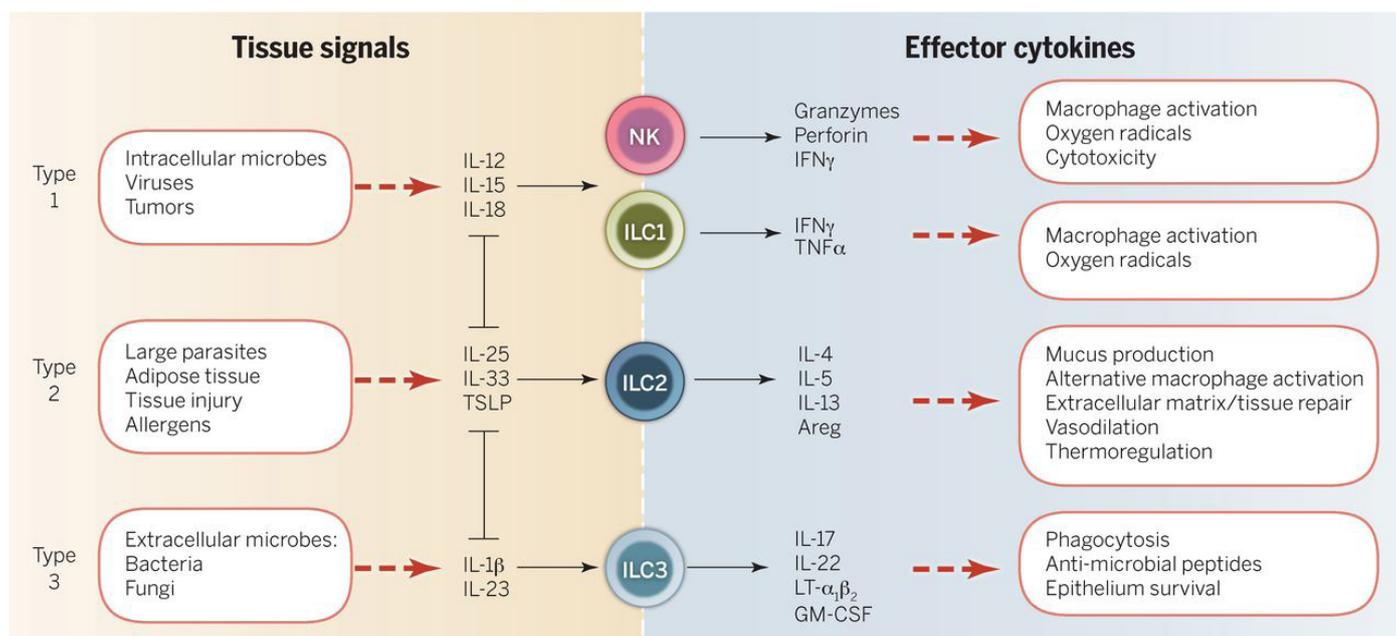


Figure 14 : Tissue-derived signals mediate ILC activation
“The biology of innate lymphoid cells”

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 Science 22 May 2015: Vol. 348, Issue 6237 DOI: 10.1126/science.aaa6566

IL-12 & IL-1 Family: Twelve plus one, not eleven plus two

IL-12 and IL-23 are both heterodimeric molecules, which share a common subunit IL-12p40¹⁴⁰, and are therefore classified as member of the IL-12 family. IL-12p40 associate with IL-12p35 to form the IL-12, which binds its cognate heterodimeric receptor composed of the two subunit IL-12Rβ1 and IL-12Rβ2. In a similar fashion, association of IL-23R with IL-12Rβ1 form the receptor for IL-23, which itself comprise the subunits IL-12p40 and IL-23p19. IL-12 and IL-23 were mentioned in the above sections as trigger for T-bet and RORγt expression, respectively.

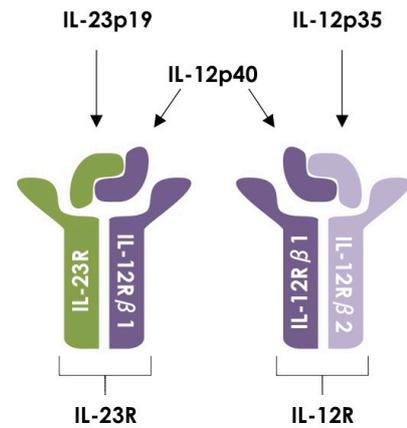


Fig 15 : IL-12 and IL-23 share the IL-12Rβ1 subunit

Unlike members of the IL-12 family, which are mainly produced by myeloid cells, both myeloid and non-hematopoietic cells, such as epithelial cells, can secrete cytokines that belong to IL-1 family¹⁴¹⁻¹⁴³. IL-1β and IL-18, like all members of the IL-1 family, are first secreted in an inactive form, which is subsequently enzymatically cleaved by caspase-1 to become bioactive. The main receptor for IL-1β is composed of the two subunits IL1-R1 and IL1-R3, while IL-18 signals through the IL-18R heterodimeric receptor, which comprises two subunits IL-18Rα and IL-18Rβ.

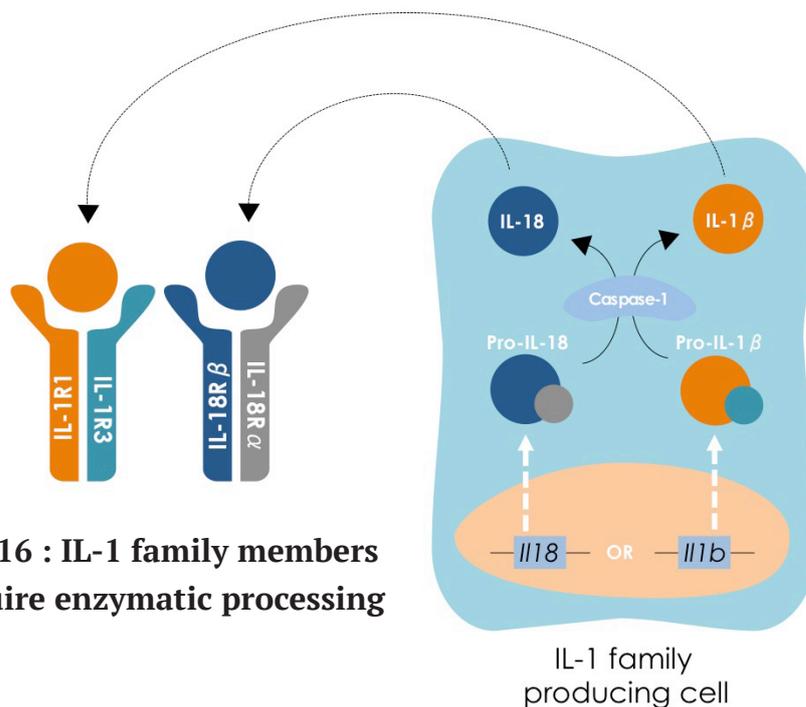


Fig 16 : IL-1 family members require enzymatic processing

Regarding T cells, an elegant study by Guo and colleagues highlighted the specific responsiveness of T^H cells toward IL-1 family members¹⁴⁴. Polarized T^H1 expressed *Il18r1* and accordingly responded to IL-18, while polarized T^H17 expressed *Il1r1* and responded to IL-1 β . Moreover, IL-1 β increased IL-23-induced ROR γ t expression by T^H17 cells, and IL-18 potentiated IL-12 induction of Tbx21 by T^H1 cells. However, stimulation with IL-1 family members only failed to elicit significant modulation of the aforementioned transcription factors, indicating that they are not the main driver for eliciting T^H lineage programs, but rather synergize with IL-12 members to increase effector functions in mature cells.

As ILC1 and ILC3 share striking similarities with T^H1 and T^H17, respectively, one can assume that they respond in a similar fashion to IL-12 and IL-1 family members.

IFN γ : An Army of one

Despite being the sole member of type II interferons, IFN- γ displays pleiotropic activities as evidenced by combined immune defects in IFN- γ deficient mice¹⁴⁵ (for reviews, see Schroder et al. 2004)¹⁴⁶. ILC1 constitutively express IL-12R and IL-18R, and produce IFN- γ production upon stimulation by IL-12 and IL-18^{101, 147}.

Originally named Macrophage Activating Factor, IFN- γ potentiate the antigen-presentation by myeloid cells by increasing the expression of both class I and class II major histocompatibility complex (MHC-I and MHC-II)¹⁴⁶, which are recognized by TCR-bearing CD8⁺ and CD4⁺ T cells, respectively. T^H1 cells also express the receptor for IFN- γ (IFNGR)⁹⁴, which is required for their full maturation. Following LPS injection, *Ifng* deficient ILC1ND efficiently migrate to lymph node, but fail to trigger T^H1 cells activation and expansion¹⁴⁸. As such, ILC1-derived IFN- γ potentially promotes antigen-presentation by myeloid cells, and expansion of adaptive T^H1 cells, demonstrating its impact in the establishment of adaptive responses (Fig. 12,17).

Moreover, IFN- γ increases iNOS (inducible Nitric Oxide Synthase) expression by myeloid cells¹⁴⁶, which catalyzes the production toxic reactive nitrogen intermediates (RINs)¹⁴⁹. Indeed, mice, in which a myeloid specific promoter drives the expression of a dominant negative form of the IFNGR, are unable to produce RINs, and subsequently fail to survive infections with *Trypanosoma cruzi* or *Leishmania major*¹⁵⁰, highlighting the role of IFN- γ in controlling myeloid-mediated clearance of these intracellular pathogens.

Although isolated myeloid cells from these mice exhibit normal IL-12p40 production, pre-incubation, or “priming”, of myeloid cells with IFN- γ lead to their enhanced production of IL-12p40, IL-12p35¹⁵¹ and IL23-p19¹⁵² production following stimulation with microbial products. During infection with *Toxoplasma gondii* and *Mycobacterium tuberculosis*, ILC1ND

are the main innate source of IFN- γ , which drives the differentiation of inflammatory monocytes into IL-12p40 producing DCs¹⁴⁸. Here, IFN- γ signals instruct myeloid cells to efficiently produce IL-12, either by “priming” or by inducing their differentiation.

With respect to IL-1 family members, prolonged exposure of macrophages to IFN- γ lead to decreased Il1b expression and IL-1 β production *in vitro*¹⁵³, while stimulation of splenocytes with IFN- γ triggers IL-18 expression, although the cellular source remains unclear.

IL-18 was originally named IFN- γ -inducing factor as it potentiated IL-12-induced IFN- γ production by T cells^{154,155}. ILC1^b isolated from IL-18 or IL-18R deficient mice fail to elicit normal IFN- γ production *ex vivo*¹⁵⁶. The defect in IFN- γ production observed in *Il18*^{KO/KO} could be restored to normal level only upon preculture of isolated ILC1^b with IL-18, but not with culture of IL-12 and IL-18, implying that IL-18 primes ILC1^b(¹⁵⁶). Still, splenic and hepatic ILC1^b compartment are normal in double deficient mice for IL-12 and IL-18^{157,158}, indicating that these cytokines instruct the function, but not the development of ILC1^b (Fig.18). Moreover, Intestinal ILC1^a were reported to display higher expression of IL-12R β ¹⁵⁶, indicating that ILC1^a potentially respond better to IL-12 compared to ILC1^b.

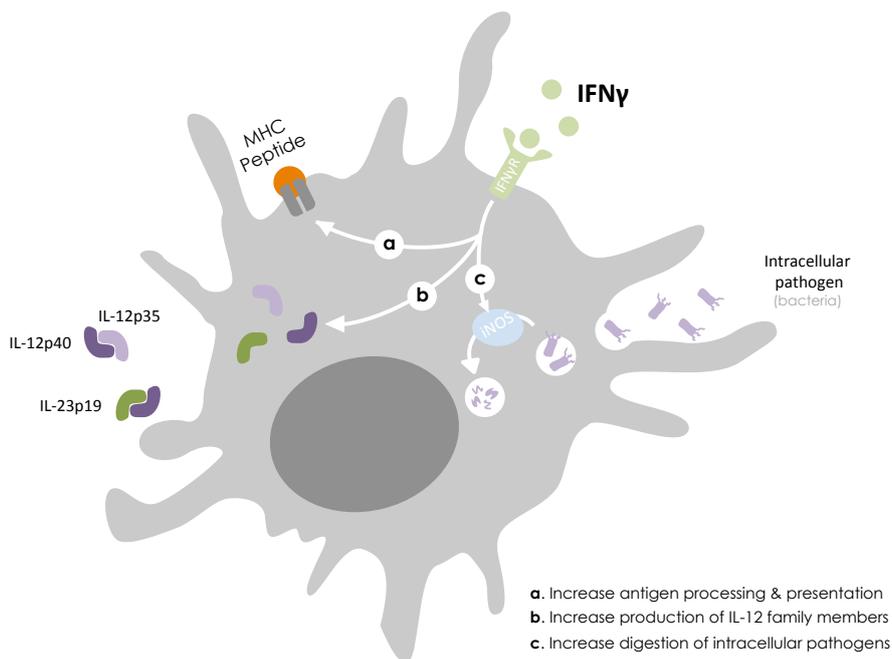


Fig 17 : Role of IFN- γ in myeloid activation

Overall, IL-12, IL-18, and IFN- γ are intertwined and provide amplifying loops in promoting T^H1 responses. As ILC1 are quick responder to IL-12 and IL-18, they provide an innate source of IFN- γ , which impacts myeloid and T cell function.

Perforin/Granzyme : Cytotoxicity is my city

CD8⁺ T cell and ILC1^b are cytotoxic lymphocytes, as they are both able to recognize infected or transformed cells, and subsequently trigger the apoptosis of the target cells. Although the recognition mechanism differs, as CD8⁺ T cell activation requires engagement of the somatically rearranged TCR while ILC1^b only require germline encoded receptors, both lymphocytes share common effector molecules to mediate their cytotoxic effect, namely Perforin and Granzymes (for review see Voskoboinik et al. 2015)¹⁵⁹. In this section, I will concisely present these molecules before going back to ILC function.

Perforins were identified as molecule that could self-assemble to generate small pores (16-22um diameter) at the surface of cells. Encoded by the *Prf1* gene, perforin is inactive at an acidic pH, and must be compartmentalized into granules to restrain its pore-forming activity. Mutation that affects the delivery of perforin into granules leads to the lysis of the lymphocytes that produces it, as the pores formed allows for liquid influx, which creates osmotic pressure and thereby disrupts the cellular membrane. Recognition of a target cells triggers Ca²⁺ influx in the cytotoxic lymphocytes, which in turn exocytose the granules content toward the target cells, a process also called degranulation.

Granules contained in cytotoxic lymphocytes comprise other molecules that are Granzymes, which are small enough to reach the cytosol of the target cells through the perforin-generated pores. Granzyme B (*Gzmb*) is the most potent pro-apoptotic molecule, as it induces apoptosis of the target-cell in less than 10 minutes through caspase activation¹⁶⁰. However, *Gzmb* deficient ILC1^b still exert cytotoxic function, which is then solely ensured by Granzyme A (*Gzma*)¹⁶⁰. Cell-death induced by Granzyme A differ from both conventional apoptosis and cell death lysis, as target cells co-cultured with *Gzmb* deficient ILC1^b exhibited cell elongation, which is morphologically distinguishable from the “beebbling” of cells that undergo conventional apoptosis. In addition, the rate of cellular death was slower compared to cell cultured with *Gzmb* proficient ILC1^b.

These findings demonstrate that ILC1^b induces target cells death through at least three mechanisms: perforin-induced cell lysis, which is a direct effect of the loss of membrane integrity of the target cells; granzyme B mediated apoptosis, which requires perforin to enter the cell and subsequently triggers caspase-induced apoptosis; and granzyme A, which induces a caspase-independent form of cell death.

ILC1^b constitutively express *Prf1* and *Gzmb*^{4, 100, 101, 147}. Eomes appear to be key in driving their expression, as it was reported to directly bind their promoter in T^H2 cells transduced with a retrovirus expressing Eomes¹⁰⁸. Interestingly, co-culture of ILC1^b with

IL-12, IL-18 and IL-15 led to increase level of Perforin, Granzyme B, T-bet, but not Eomes when compared to IL-15 alone¹⁶¹. T-bet deficient ILC1^b display lower IFN- γ production in response to IL-12 and IL-18, and reduced cytotoxicity toward target cells¹⁰⁰. Moreover, IL-12 alone, or in combination with IL-18 increase *Tbx21* transcripts in ILC1^b, while IFN- γ do not. Remarkably, T-bet binds the promoter *Ifng* and *Gzmb*, but not *Prf1*. Similarly to IFN- γ expression, IL-18 potentiated IL-12 induced ILC1^b mediated cytotoxicity. Stimulation with IL-18 upregulates transcripts for *GrzmB* and *Prf1*, but not the proteins GranzymeB and Perforin^{147,156}, indicating that IL-18 promotes cytotoxic functions by increasing the pool of mRNA transcripts (Fig.18). Eventually, transcripts are translated into proteins upon another stimulus such as IL-12, pointing out to a role for IL-18 regulation of cytotoxic activity in ILC1^b. Altogether, these results point the critical role of Eomes, which expression appear stable, in controlling perforin and granzymes expression in resting ILC1^b, while T-bet reacts to IL-12 and IL-18 and subsequently tune ILC1^b effector function by regulating the expression of *Gzmb* and *Ifng*.

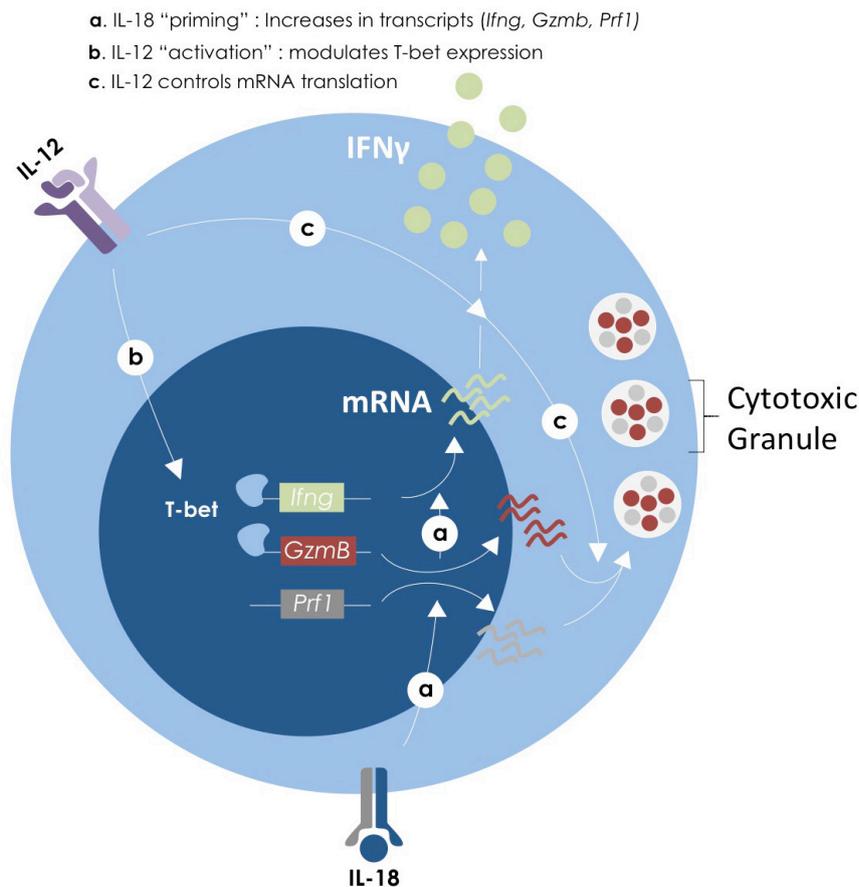


Fig 18 : IL-12 and IL-18 regulates T^H1 associated genes

GM-CSF : GM-etting CeriouSF

Encoded by the *Csf2* gene, Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) was first reported as a soluble factor that promoted the expansion of mixed colony of granulocytes and macrophages from mouse bone marrow cells¹⁶², and has been widely used to generate BMDCs (bone marrow derived DCs)¹⁶³, which are in fact an heterogeneous population¹⁶⁴.

Regarding the T^H source of GM-CSF, stimulation of T^H17 cells with IL-23 increases *Csf2* transcripts and subsequent GM-CSF production¹⁶⁵. While *Il12rb1* is crucial for GM-CSF production by T^H17 cells, ROR γ t and IL-12R β 2 are both dispensable, as ROR γ t or IL-12R β 2 deficient T^H cells still exhibit GM-CSF upon in vitro stimulation¹⁶⁵. Remarkably, stimulation with IL-1 β either potentiates IL-23 effect on T^H17-GMCSF production or trigger GM-CSF production in T^H1 polarized cells *in vitro*¹⁶⁶. As such, GM-CSF does not appear as a lineage-restricted cytokine, but is rather produce in diverse settings in response to IL-23 and/or IL-1 β .

GM-CSF can be produced by both ILC1 and ILC3^{56, 101, 142, 167, 168} (Fig.19). Splenic ILC1^b with IL-18 or IL-12 induces their production of GM-CSF¹⁶⁹. *Ex vivo* stimulation of ILC3 with IL-1 β , but not with IL-23, triggered GM-CSF production¹⁴². Except for the GM-CSF production by IL-12 observed in ILC1^b, which could be explained by activation of IL-12R β 1 downstream pathway, members of the IL-1 family therefore control GM-CSF production both in ILC1^b and ILC3^{142, 167}. However, ILC1^b are poor producer of GM-CSF upon *ex-vivo* stimulation with PMA/ionomycin¹⁰¹, which contrasts with ILC1^a that steadily produce GM-CSF in the same conditions, indicating that ILC1^a could present an early source of GM-CSF production.

Similarly, ILC3 are the main source of intestinal GM-CSF in steady state upon *ex vivo* stimulation with PMA/Ionomycin¹⁶⁷. As such, the level of GM-CSF in *Rorc* deficient mice, which lack ILC3, approximates those of *Csf2* deficient mice¹⁶⁷. *Csf2* deficiency perturbs the intestinal myeloid compartment, exemplified by a marked reduction of both DCs and macrophages. ILC3 derived GM-CSF therefore appears to maintain myeloid cell homeostasis in the intestine. In turn, myeloid provided a source of IL-1 β upon sensing of the intestinal microbiota, demonstrating the crosstalk between ILC3 and myeloid cells in mucosal tissue¹⁶⁷ (Fig.19).

In another context, adoptive transfer of *Csf2* deficient splenic ILC3 in RAG^{KO/KO} *Il2rg*^{KO/KO} mice failed to restore the neutrophil compartment present in RAG^{KO/KO} mice, while their *Csf2* proficient counterpart did, bringing more evidence for the role of GM-CSF derived ILC3 in myeloid homeostasis¹⁷⁰ (Fig.19).

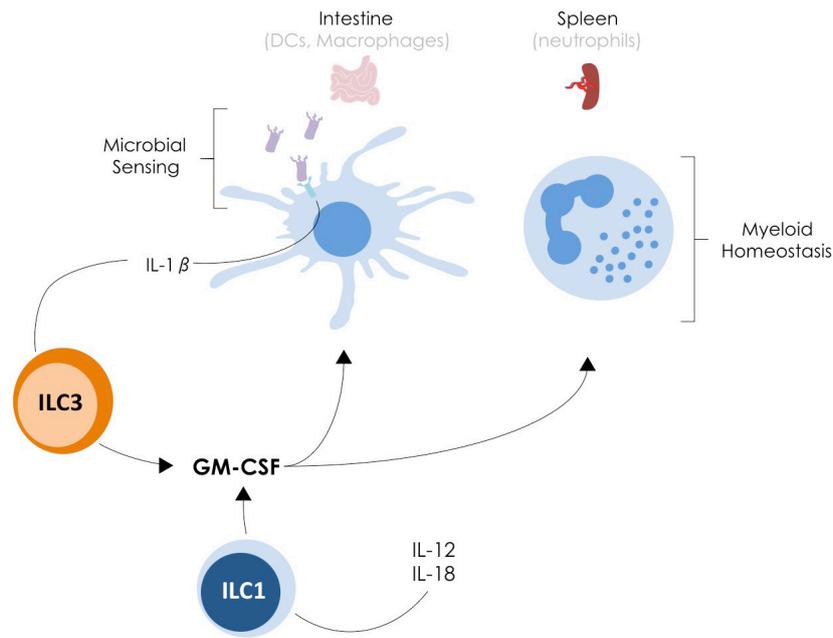


Fig 19 : ILC-derived GM-CSF control myeloid homeostasis

IL-17 : Sweet seventeen

IL-17 family of cytokines regroups 6 members (A to F)(for review see Korn et al. 2009)¹⁷¹. In this section, I will focus on IL-17A and IL-17F.

In T^H cells, six days culture of activated T^H cells with IL-23 induces *Il17a* and *Il17f* transcripts¹⁷², while *Il23r* deficiency led to a marked decrease in IL-17A production¹⁷³. Consistent with a role for IL-23 in sustaining *Rorc* expression¹¹⁶, *Rorc* deficient T^H cells fail to elicit normal IL-17A production¹¹⁵, stressing the importance of RORγt expression in T^H17 function.

Both IL-17A and IL-17F, which are encoded by *Il17a* and *Il17f*, respectively, bind the heterotrimeric receptor IL-17RC(1:2)IL-17RA¹⁷⁴, encoded by *Il17rc* and *Il17ra*, respectively (Fig.20). Transcripts for *Il17ra* are ubiquitous, but highly enriched in hematopoietic cells¹⁷⁵, while a splice form of *Il17rc* is specifically expressed in colonic epithelial cells (CECs)¹⁷⁴, suggesting that these subunits combine with other receptor(s) and deliver distinct cell-type specific signals. For instance, either IL-17A or IL-17F in vitro stimulation of CECs elicits the production of CXCL1 (a chemokine that induces neutrophil recruitment), IL-1β release, while only IL-17A stimulates T cells or peritoneal macrophages to produce the same mediators¹⁷⁶. IL-17A proved itself a more potent



Fig 20 : Schematic representation of the IL-17R

inducer of the anti-microbial peptide (AMP) Lipocalin-2 production in CECs compared to IL-17F, which itself was more potent than IL-17A in triggering the secretion β -defensin 3, another AMP¹⁷⁶ (Fig.21).

Although neither IL-17A nor IL-17F are required for the clearance of *Citrobacter rodentium*, an enteropathogenic bacteria causing substantial intestinal inflammation that attach to intestinal epithelial cells, deficiency in either *Il17f* or *il17a* leads to increased colonic hyperplasia and bacterial burden¹⁷⁶. Increased pathology correlated with decreased levels of AMPs such as β -defensins 1, 2 and 3, indicating that IL-17A and IL-17F participate in the defense to *Citrobacter rodentium* in the intestine, but not its clearance.

In a model of Dextran Sodium Sulfate (DSS) induced colitis, IL-17F level was equivalent between RAG and WT mice following stimulation of intestinal lymphocytes with IL-23¹⁷⁷. In the same condition, IL-17A was reduced three-folds in RAG mice compared to WT. These results indicate that RAG independent cells produce preferentially IL-17F, and few IL-17A, following IL-23 stimulation.

No study reported IL-17A or IL-17F expression by neither NKp46⁻ nor NKp46⁺ ILC3^{126, 178}, while CD4⁻ and CD4⁺ ILC3 are responsible for innate IL-17A production^(65, 126). Nonetheless, IL-23 stimulation is a poor inducer of IL-17A production by CD4⁻ and CD4⁺ ILC3, and assays often combined IL-23 stimulation with PMA ionomycin alone to assess intracellular IL-17A production, a strong trigger that do not always reflect biological conditions.

The use of IL-17A blocking antibodies in RAG mice proved itself useful to address ILC3 function in vivo. Mice fed with high-fat diet (HFD) become obese, and subsequently develop airway hyper-responsiveness (AHR), which is mimicked upon IL-1 β intra-nasal treatment¹⁷⁹. *Il17a* deficient mice were not responsive to IL-1 β , while blocking of IL-17A in IL-1 β treated RAG mice abrogated AHR. As CD4⁻ ILC3 were the sole source of IL-17A in RAG treated with IL-1 β , these results suggest that CD4⁻ ILC3 can respond to IL-1 β by secreting IL-17A *in vivo*¹⁷⁹.

In another setting, lung infection with *Klebsiella pneumoniae*, an extracellular bacteria, is fatal in RAG mice depleted with anti-IL-17A antibody¹⁸⁰. As myeloid cells isolated from the lung of *Il17a* deficient mice display a lower intracellular load of bacteria, it was proposed that that CD4⁻ ILC3-derived IL-17A promotes the uptake of extracellular bacteria and therefore facilitate their clearance.

Moreover, *Il17ra* deficient mice or ILC1 depleted mice are highly susceptible to infection with the fungus *Candida albicans*¹⁸¹, while RAG mice exhibited similar fungal load with WT mice. Transfer of WT ILC1^b in RAG^{KO/KO} *Il2rg*^{KO/KO}, but neither *Il17ra* nor *Csf2* deficient ILC1^b, reduced fungal load observed in control mice, implying a role for *Il17ra* signaling in controlling GM-CSF production by ILC1^b. Upon co-culture, ILC1^b potentiated neutrophils activity, which was abolished by blocking with an anti-GM-CSF antibody.

Although the exact source of IL-17A/F production in RAG mice was not addressed, CD4⁻ ILC3 are an appealing surrogate, indicating that they might influence neutrophil activity through the triggering of GM-CSF by ILC1 through IL-17A/F production¹⁸¹.

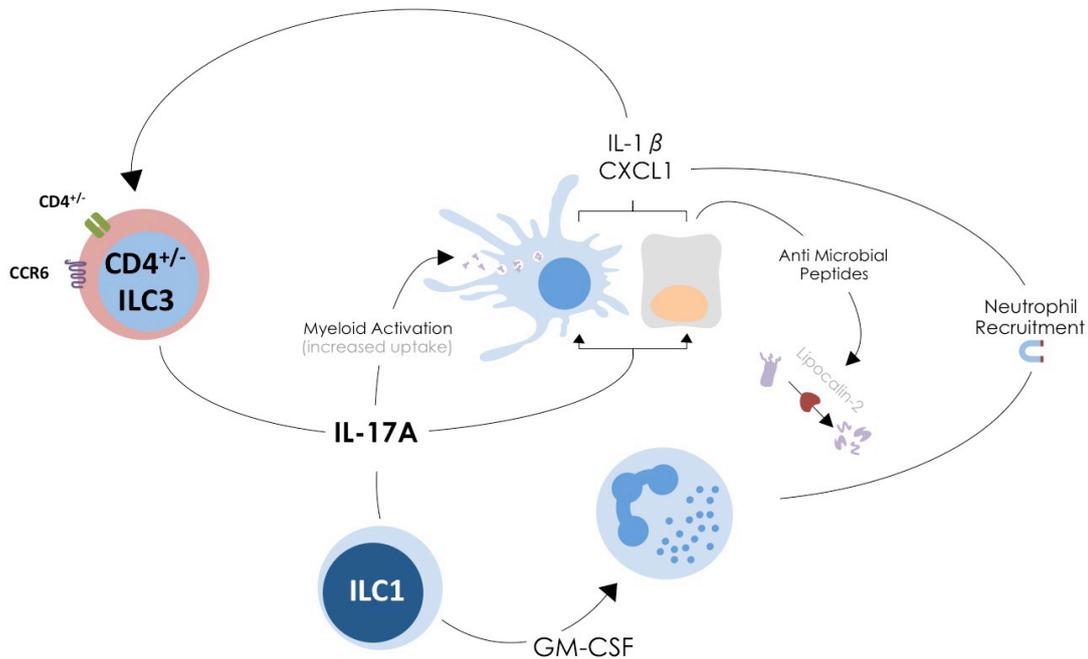


Fig 21 : ILC-derived IL-17 promotes antimicrobial defenses and myeloid activation

IL-22 : Border control

IL-22 production maintains homeostasis at mucosal sites. In mice, IL-22 deficiency leads to increased susceptibility to pulmonary^{182, 183} or intestinal bacterial infection¹⁸⁴ and impaired epithelial regeneration¹⁸⁵⁻¹⁸⁷.

IL-22 sequentially binds IL-22Rα1 and recruits IL-10Rβ2 to transduce signals¹⁸⁸. While IL-10Rβ2 expression is ubiquitous, IL-22Rα1 expression is restricted to non-hematopoietic cells. In mice, epithelial cells from the lungs¹⁸³, the small^{141, 143, 189} or large intestine¹⁸⁴, pancreatic β-cells¹⁹⁰, keratinocytes and skin fibroblasts as well as thymic epithelial cells¹⁸⁶ harbor *Il22ra1* transcripts, the gene encoding IL-22Rα1, and subsequently respond to IL-22. *Ex vivo* stimulation with IL-22 lead to the upregulation of transcripts that encode AMPs such as *Reg3g*, *Reg3b*, *S100A9*, *S100AB* by mouse colon¹⁸⁴ or lipocalin-2 and mucin by tracheal epithelial cells¹⁸³, *Il18* by small-intestine epithelial cells¹⁴³ or extracellular matrix components like fibronectin and collagen by skin fibroblasts¹⁸⁵. These data indicate that IL-22 ensures the protection and reparation at mucosal sites by regulating a wide set of genes.

ROR γ t expressing lymphocytes are the only source of IL-22. T^H17 cells efficiently produce IL-22 upon IL-23 stimulation^{184, 191}, a production potentiated by IL-1 β ¹⁶⁶. In isolated ILC3ND, IL-22 production is triggered by either IL-23 or IL-1 β alone^{142, 192, 193}. In addition to IL-23, myeloid produce TNF-Like ligand 1A (TL1A), which potentiates IL-1 β or IL-23-driven ILC3ND IL-22 production¹⁴².

One report stressed the role of the IL-1 α , another IL-1 family member, in inducing IL-22 expression by sorted ILC3^{ND(141)}. Rotavirus infected intestinal epithelial cells were the sole source of IL-1 α , which proved itself as potent as IL-1 β in activating ILC3 to secrete IL-22, demonstrating that non-hematopoietic cells also activate ILC3 (Fig.22).

As a matter of fact, IL-22 expression requires both ROR γ t and the aryl hydrocarbon receptor (encoded by *AhR*)¹⁹⁴, a NR that strictly exerts its transcriptional regulation upon binding with ligands like nutrient-derived xenobiotic or environmental toxins (for review see Zhou 2016, and Stockinger et al. 2014^{195, 196}). *AhR* deficient mice exhibit a clear defect in IL-22 production, both by T^H17¹⁹⁴ and ILC3^{197, 198}. ROR γ t has been proposed to interact with *AhR* and facilitate their binding to the *Il22* locus¹⁹⁷.

Indole-3-aldehyde (IDO1, encoded by *Ido1*) is the main enzyme responsible for catabolising dietary derived tryptophan, and is highly expressed by intestinal myeloid cells. *Ido1* deficient mice exhibit increased tryptophan availability in the gastrointestinal tract, which leads to the expansion of bacterial species such as *Lactobacillus reuteri*¹⁹⁹. Remarkably, these bacteria produce a metabolite, Indole-3-aldehyde (IAld), which activates AhR-mediated transcription. Stimulation of sorted NKp46⁺ ILC3 with IAld elicited IL-22 production¹⁹⁹, demonstrating that bacterial-derived metabolites directly activate NKp46⁺ ILC3, presumably in an AhR dependent manner.

Reciprocally, ILC3ND also influences substrate availability for gut microbiota^{189, 200}. α (1,2)fucosyltransferase, encoded by *Fut2*, is required for the addition of fucose residues at the surface of intestinal epithelial cells, which, upon shedding, is used by commensal bacteria as a sugar source. *Il22* and *Rorc* deficient mice display possess lower *Fut2* transcripts. Treatment of RAG mice with a blocking IL-22 antibody results in decreased epithelial fucosylation, pointing out to a role for ILC3-derived IL-22 in controlling fucosylation¹⁸⁹. Subsequent to the modification in sugar availability, gut microbiota from *Fut2* deficient mice switched their metabolic profile²⁰⁰, which rendered *Fut2*^{KO/KO} mice more susceptible to infection with enteric pathogens such as *Salmonella typhimurium*¹⁸⁹ and *Citrobacter rodentium*²⁰⁰.

Similarly, IL-22 deficient mice succumb rapidly upon *Citrobacter rodentium* infection, as they failed to produce AMP in response to infection. Indeed, survival was improved following injection with the AMP RegIII γ ¹⁸⁴, which is induced by IL-22 in colonic epithelial cells. RAG mice are able to control the early stages of *Citrobacter rodentium*

infection, but strictly require adaptive immunity to completely clear the pathogens^{64,184}. Still, mice with conditional deletion of AhR in *Rorc* expressing cells display decreased survival²⁰¹, either in a T cell sufficient or deficient background, pointing out a role for IL-22 producing lymphocytes in bacterial control at intestinal sites.

Injection of IL-22 blocking antibody increases the bacterial burden in the lungs of *Klebsiella pneumoniae* infected mice¹⁸³, which subsequently succumb earlier than untreated mice. Another pulmonary pathogen, *Streptococcus pneumonia*, elicits strong IL-22, IL-17A and IL-17F production by CD4- ILC3, both in WT and RAG mice¹⁸². Concomitant intravenous injection with flagellin, a microbial derived product, potentiated cytokine production by CD4- ILC3, and largely increased survival of *Streptococcus pneumonia* infected mice. However, flagellin protective effect was abrogated either in IL-22 deficient mice or mice treated with an IL-22 antibody.

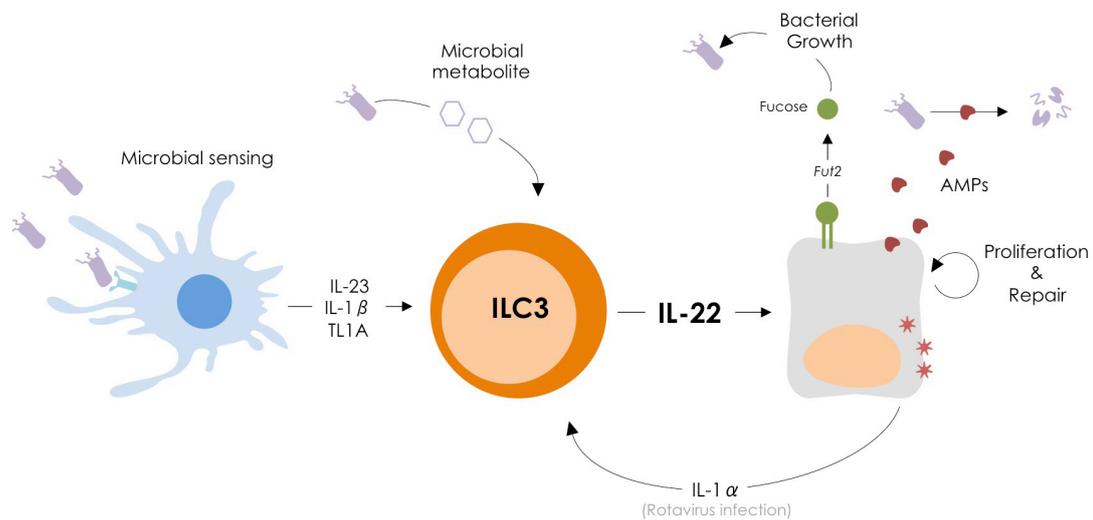


Fig 22 : ILC-derived IL-22 maintain mucosal integrity

Overall, mucosal homeostasis is tightly linked to IL-22 production. Non-hematopoietic cells respond and instruct lymphocyte derived IL-22, which can be activated directly or via myeloid cells by microbial derived products and in turn promote defense against pathogens and tuning of the gut flora.

Chapter III

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ILC1 to 3

This chapter aims to recapitulate important features of ILC1 and ILC3, some of which were already addressed in the above sections. I will first stress biological functions implicating NKp46, a surface marker shared by ILC1 and a subset of ILC3. Then, I will briefly address ILC1 diversity before focusing on ILC3.

NKp46 : The marker of beast

Screening for antibodies that could modulate ILC1^b cells (also known as NK or Natural Killer) cytotoxicity is a method of choice to identify potential therapeutic targets. A 46kD surface protein (p46) was identified in 1997 as constitutively expressed by ILC1^b, and subsequently named NKp46. Encoded by *Ncr1*, and also known as Mar-1 or CD335, NKp46 was proposed as a unifying marker to identify ILC1^b cells throughout species. In mice, its steady-state expression is restricted to ILC1^{34,132,132} and a population of ILC3^{123,124,126}, a finding that was emphasized by NKp46 Fate-mapping studies²⁰².

However, NKp46 ligand(s) remain(s) unknown. To address this question, a protein composed of the extracellular domain of NKp46 linked to an Immunoglobulin domain (NKp46-Ig) was generated²⁰³. Following infection with lymphocytic choriomeningitis virus, mice deficient for the type I IFN receptor, *Ifnar*^{KO/KO}, CD8⁺ T cells bore higher amounts of NKp46-ligand on their surface²⁰⁴. Consequently, CD8 heightened susceptibility towards ILC1ND-cell mediated killing affected the generation of the memory pool in a *Ncr1* dependent manner²⁰⁴. Increased expression of NKp46 ligands by adipocytes has also been associated with insulin resistance²⁰⁵. As NKp46-cross-linking triggered ILC1ND cells activation, mice that underwent high fat diet had increased IFN- γ production in visceral adipose tissue, which in turn promoted the polarization of pro-inflammatory macrophages²⁰⁵.

NKp46-Ig also recognizes human monocytes infected with *Mycobacterium tuberculosis*²⁰⁶. Immunoprecipitation with NKp46 antibody identified vimentin, an intracellular protein required for cytoskeleton architecture, as a putative ligand. In a similar fashion, incubation of cells previously infected (either with Sendai or Influenza virus) with hemagglutinin-blocking antibodies prevented NKp46-Ig binding. Both hemagglutinins binds sialic acid that are shed by the viral Neuramidase, which, when inhibited, increases NKp46-Ig binding²⁰⁵. It was therefore proposed that NKp46 binds Hemagglutinin both in a sialic acid dependent and independent manner. As such, *Ncr1* deficient mice succumb to influenza infection, highlighting a role for *Ncr1*-mediated recognition in viral infection²⁰⁷.

Lower surface NKp46 expression in human ILC1^b cells co-cultured with influenza-infected cells is associated with greater cytotoxic potential²⁰⁸. Whether this downregulation reflects engagement of the receptor, blocking of the epitope by interaction with HA or transcriptional regulation remains to be addressed. Surface NKp46 was also reported to be modulated following mouse cytomegalovirus infection²⁰⁹. Sixty days after transfer into *RAG*^{KO/KO} *Il2rg*^{KO/KO} mice, NK cells exhibited lower amount of surface NKp46 and enhanced activity seven days post-infection when compared to non-infected mice.

ILC1 day

ILC1 regroups ILC1^a, ILC^b and ILC^{ab}, all of which respond upon IL-12 and/or IL-18 stimulation by producing IFN- γ . ILC1 all bear surface expression of NKp46 and NK1.1, which is the orphan c-type lectin-like receptor that permitted identification of ILC1^b for years^{210, 211}. ILC1 also all harbor CD122 that allows for IL-15 responsiveness and their homeostasis. Despite these unifying features, ILC1 exhibit differential function, ontogeny and localization.

ILC1^b circulate through the blood stream and constantly replenish organs, while ILC1^a and ILC1^{ab} are tissue resident. Moreover, both T-bet and GATA3 are required for Eomes-independent ILC1^a generation, but only impacts the maturation of Eomes-dependent ILC1^b. Based on distinct dependency of ILC1^a and ILC1^b toward transcription factors, different origin and tissue residency, it is only fair to assume that ILC1^a and ILC1^b represent distinct lineages. Replacing ILC1^{ab} appears complicated, as few reports clearly addressed their ontogeny, which will be discussed in the ILC1^{ab} section.

ILC1b : “Natural (Born) Killer”

ILC1^b represent the prototypic ILC, and was used as a reference upon the discovery of other ILC subsets. Extensive reviews describe their development²¹², the activating/inhibiting receptors involved in target cell recognition by ILC1^{b(213, 214)} and their subsequent activation^{215, 216}, as well as their tissue distribution²¹⁷. Regarding ILC1^b precursors, the discrepancies between the markers used in the different studies are hard to reconcile, especially in view of the discovery of novel ILC1 lineages. Therefore, I will succinctly present ILC1^b development, which will hopefully be clarified in future studies.

The antibody clone for NK1.1 fails to recognize epitopes in all mouse strain²¹¹, research groups ofent relied on the coexpression of CD122 and CD49b²¹⁸, which is recognized by the DX5 antibody clone, to identify ILC1^b. As mentioned above, restricted precursors for ILC1^b reside in the bone marrow and are characterized by the lack of lineage marker, including NK1.1, but express CD122. BM Lin⁻ CD122⁺ comprises a portion of NK1.1⁻ CD49b⁺⁽⁷⁴⁾, indicating that CD49b expression precedes that of NK1.1, which is then followed by NKp46 upregulation²⁰².

Regarding the accepted scheme of ILC1^b maturation, the most immature stage is contained within the CD27⁺ fraction²¹⁹, which is in fact an heterogenous population containing ILC1^b and ILC1^a. Nonetheless, CD27⁺ acquire CD11b (CD27⁺ CD11b⁺ or DP stage), and subsequently lose CD27 and only retain CD11b expression (CD11b⁺). This maturation process is associated with an increase in cytotoxic function, a loss of proliferative potential and acquisition of fully mature Ly49 repertoire, which will dictate ILC1^b responsiveness²²⁰. The final stage of ILC1^b maturation is characterized by expression of the exhaustion marker KLRG1¹⁵⁷, an inhibitory receptor associated with cellular senescence.

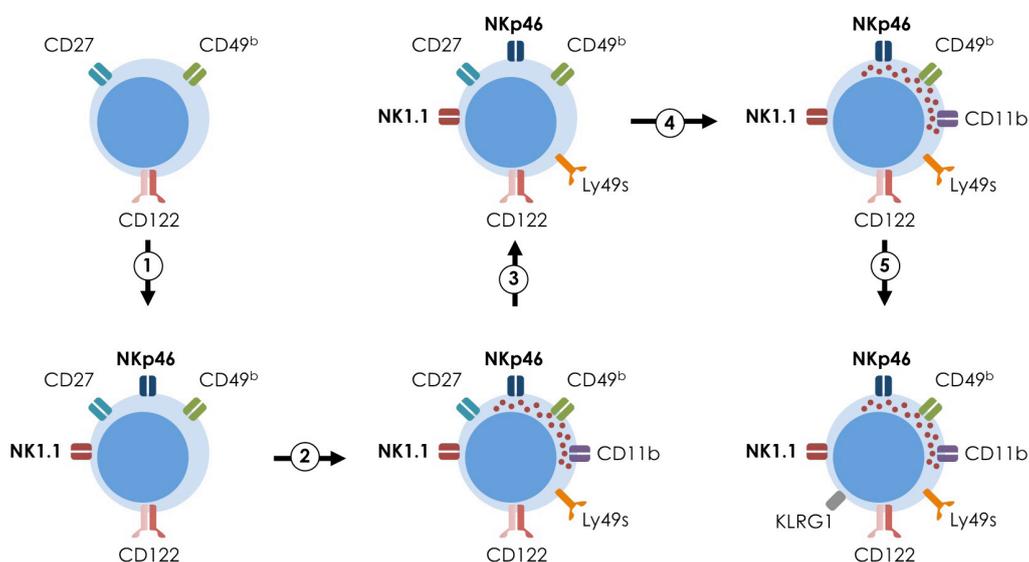


Fig 23 : ILC1^b maturation is a sequential process

Immature ILC1^a

ILC1^a were originally classified as “immature” NK cells generated during fetal hematopoiesis¹⁵⁸, characterized by constitutive surface expression of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)²²¹.

TRAIL is recognized by surface Death Receptors (DR), among which DR5 directly triggers apoptosis upon binding²²². As DR5 expression is induced in response to DNA-damage²²³ or ER stress²²⁴, some tumors are susceptible to TRAIL-mediated apoptosis. In the liver, TRAIL expression is restricted to ILC1^a in steady state^{100, 101, 132, 221}, and is required for tumor surveillance. Indeed, isolated hepatic lymphocytes from *Prf1* deficient mice efficiently killed L929 cells *in vitro*²²¹, while blocking of TRAIL diminished killing efficiency, indicating that ILC1^a kill tumor cells in a perforin-independent TRAIL-dependent manner. As TRAIL deficient mice still harbor ILC1^{a(221)}, TRAIL is therefore dispensable for their generation.

Although ILC1^a were not affected by the lack of IFNGR, but totally lost TRAIL expression²²¹, indicating that IFN γ signaling is directly or indirectly required for TRAIL induction. Remarkably, splenic ILC1^b upregulated TRAIL expression upon injection of IFN γ *in vivo*¹⁵⁸, or following stimulation with IL-12 and IL-15 *in vitro*²²⁵, indicating that TRAIL expression can be induced in ILC1, which will be discussed later.

ILC1^a distinguish from ILC1^b by their expression of the integrin CD49a and the chemokine CXCR6¹³², both of which are also expressed by NKT cells, and by their lack surface expression of CD49b, CD11b, Ly49s as well as *Prf1*, *Grzma* and *Grzmb* transcripts^{101, 108, 132}, which would have represented an “immature” ILC1^b phenotype. In neonates, all ILC1ND are TRAIL⁺ DX5⁻, both in the spleen and the liver²²¹, which were all absent in *Tbx21* deficient mice from d0 to day7¹⁰⁰. In view of their dependency on T-bet and their surface phenotype, these cells are most likely ILC1^a (Fig.23).

ILC1^{ab} – Real inbetweenener or just a faker ?

Analysis of Nfil3 deficient mice, which lacks all ILCs, revealed that ILC1^{ab} still develop in the uterine decidua^{226,227} and the salivary gland (SG)^{32,34}. ILC1^{ab} are identified as CD49a⁺ CD49b⁺ Eomes⁺ T-bet⁺, reside in the tissue similarly to ILC1^a, and are still detected in *Tbx21* deficient mice³⁴, like ILC1^b. Unlike ILC1^a and ILC1^b, ILC1^{ab} also expressed the retention marker CD103, which marks tissue-resident CD8⁺ memory T cells²²⁸ and myeloid cells²²⁹.

In SG ILC1^{ab}, targeted invalidation of Eomes in NKp46 expressing cells³⁴ induced the loss of the ILC1^b associated marker CD49b, rendering ILC1^{ab} virtually indistinguishable from ILC1^a. This result is reminiscent of Eomes invalidation¹⁰⁰ in isolated hepatic ILC1^b, and brings further proof that Eomes instructs CD49b expression in ILC1. Unfortunately, the impact on the Ly49 repertoire has been addressed, as SG ILC1^{ab} express Ly49s³⁴. Because Eomes expression precedes that of NKp46, to confirm whether ILC1^{ab} depend on Eomes for their generation or their homeostasis would require driving Eomes-deletion by an upstream marker.

Remarkably, invalidation of TGFβ receptor³² in NKp46 expressing cells totally shifted the proportion of the SG ILC1 compartment from ILC1^{ab} to ILC1^b. In addition, isolated splenic ILC1^b cultured in the presence of TGFβ and IL-2³² led to a decrease in Eomes expression and the upregulation of ILC1^a associated markers, like TRAIL, CD49a but also CD103, and therefore phenotypically resembled ILC1^{ab}. The acquisition of ILC1^{ab} was abrogated upon TGFβR deletion, further demonstrating that this process intrinsically required TGFβ (Fig.24).

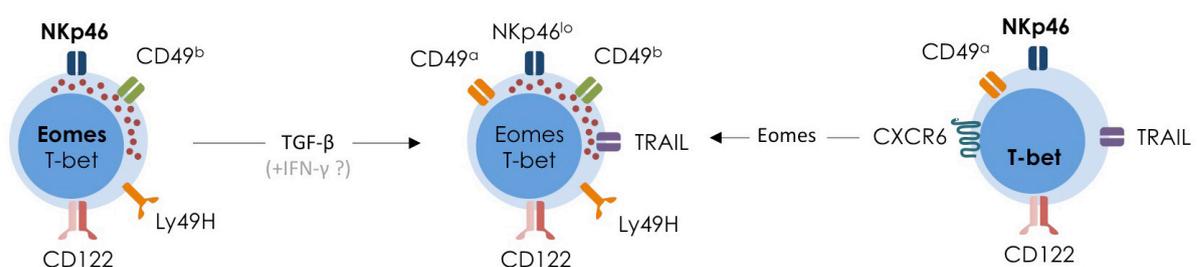


Fig 24 : Relative role of TGFβ and Eomes in the establishment of ILC1a versus ILC1^b features

Autocrine TGFβ secretion promotes tumor growth²³⁰. An ILC1 population characterized as CD103⁺ CD49a⁺ Eomes⁺ GranzymeB⁺ CD127⁻, a phenotype that overlaps with ILC1^{ab}, resided in mammary tumors and was still present in IL-15 and Nfil3 deficient mice²³¹. Although the biological function of ILC1^{ab} was not addressed in these settings, this study stresses the presence of ILC1^{ab} in anti-tumoral responses.

During MCMV infection, TRAIL⁺ CD49b⁺ ILC1ND accumulates in the salivary gland, which presumably are ILC1^{ab} that expand *in situ*²³². ILC1^{ab} interacted with T^H cells in SG histological cut, while TRAIL deficient mice exhibited increased numbers of DR5⁺ T^H cells, indicating that ILC1^{ab} induced the death of T^H cells in a TRAIL dependent manner. Remarkably, TRAIL or DR5 deficient mice exhibited lower SG titers of MCMV, indicating that ILC1^{ab}-mediated T^H cell death dampened viral control. However, infected TRAIL deficient mice had higher seric antibodies directed against host molecules and subsequently developed autoimmune disease. Unfortunately, this study did not further characterize the different actors implicated in this setting. Still, these results indicate that ILC1^{ab}-mediated control of T^H cells limits auto-immunity in a TRAIL dependent manner.

Finally, *Nfil3* deficient mice totally lack ILC1 in SG in our hands. Consistent with this observation, the fact that TGFβ imprint ILC1^b to switch to an ILC^{ab} phenotype and that Eomes dependency has not been fully addressed, it appears to me that residuals ILC1^b present in *Nfil3* deficient mice first reach the SG, then, in contact with the large amount of TGFβ present in the tumor or SG environment, switch to an ILC1^{ab} phenotype and stop requiring IL-15 and *Nfil3* for their maintenance²³³. This hypothesis remains purely speculative, and will hopefully be addressed in future studies.

ILC3: Prying open my third eye.

RORγt⁺ tissue-resident Group 3 innate lymphoid cells are CD127⁺ CD117⁺ and produce IL-22 upon IL-23 and/or IL1β stimulation. ILC3 comprises at least two distinct lineages, based on ontogeny, transcription factor dependency and function.

Lymphoid Tissue inducer: Initiate

CD4⁺ ILC3 were the second ILC subset to be identified after ILC1^b. Both CD4⁻ and CD4⁺ can produce IL-17 and IL-22, which roles were already discussed above. In addition, they orchestrate the generation of secondary lymphoid organs (SLOs), which foster a microenvironment indispensable for the establishment of adaptive immune responses, a process that has been extensively studied (for reviews, see Mebius 2003, Van de Pavert & Mebius 2010)^{234, 235}. I will therefore roughly present the mechanisms involved in this process, before addressing other CD4⁻ and CD4⁺ ILC3 features and function.

The two main SLOs are intestinal Peyer's patches (PP), which are largely populated by B cells, and Lymph Nodes (LN), which comprise mostly T cells. SLOs organogenesis is onset as early as E13.5 by CD4⁺ ILC3, which interact and activate stromal cells through the respective expression of Lymphotoxin $\alpha 1\beta 2$ (LT $\alpha 1\beta 2$) and its cognate receptor Lymphotoxin- β R. The reciprocal activation of CD4⁺ ILC3 and stromal cells lead to the secretion of chemokines and upregulation of integrins, which allows both for their own clusterisation and for the recruitment of other hematopoietic cells, i.e. DCs, B, and T cells.

As a matter of fact, LT $\alpha 1\beta 2$ by isolated CD4⁺ ILC3 can be induced by IL-7 *in vitro*²³⁶. Injection of pregnant mice with a blocking antibody for CD127 abrogates PP formation²³⁷, while mice overexpressing IL-7 display higher transcripts encoding LT $\alpha 1\beta 2$ ²³⁸, and a subsequent aberrant increase in the numbers of Peyer's patches. In addition, supplementation of culture media with IL-7 decreased the apoptosis of CD4⁺ ILC3²³⁸, demonstrating that IL-7 and its cognate receptor, CD127, are mandatory for CD4⁺ ILC3 function and homeostasis.

With respect to CD127 and CD117 expression in ILC3, two populations can be discriminated¹²⁶ : CD127^{hi} CD117^{hi} and CD127^{int} CD117^{int}. The majority of CD127^{hi} CD117^{hi} is CCR6⁺, which includes CD4⁺ ILC3. CCR6 binds CCL20, and allow for the homing of B cell in PP²³⁹ and the recruitment of T^H17 at inflammatory sites²⁴⁰. In intestinal ILC3, CCR6 is a surrogate marker used to characterize the lineage associated with lymphoid tissue inducing function. Indeed, transfer of CCR6⁺ ILC3, which include CD4⁻ and CD4⁺ ILC3 (see nomenclature), in RAG^{KO/KO} *Ii2rg*^{KO/KO} fail to generate other ILC3 subsets¹⁷⁸, indicating that they are not precursor for other ILC3 subset, but rather a distinct lineage. As mentioned above, CD4⁺ ILC3 are all CCR6⁺ in adult mice. However, fetal ILC3 display an heterogeneous distribution in CD4 and CCR6 expression (personal observation), indicating that CD4 acquisition do not reflect a maturation process, and should strictly be used in combination with CCR6.

Early characterization identified several markers for CD4⁻ and CD4⁺ ILC3, among which CD25 and MHC-II²⁴¹ are required to promote intestinal homeostasis^{242, 243}. Targeted deletion of class II molecules in ILC3 cells led to the abnormal expansion of colonic T^H17 cell that reacts to microbiota derived products, which subsequently generates intestinal inflammation²⁴². Upon incubation with exogenous peptides, isolated MHC-II⁺ ILC3 were able to process and present MHC-II-peptide complex as efficiently as professional antigen presenting cells²⁴². MHC-II⁺ ILC3 cells constantly migrate in the intestinal draining LN (mesenteric LN, or mLN), both in steady state and infection²⁴⁴. CD4⁻ and CD4⁺ ILC3 express CCR7 and migrate toward CCL21 *in vitro*, which is produced by lymphatic endothelial cells *in vivo*. In addition, CCR7 deficient mice lack CD4⁻ and CD4⁺ ILC3, indicating that CCR7 was required for their migration to LN²⁴⁴. Also known as IL2-R α , CD25 is encoded by the *Il2ra* gene, and associates with CD122 and CD132 to form the high affinity receptor for IL-2, but is also able to bind IL-2 on its own (for IL-2, see review Boyman and Sprent 2012)²⁴⁵. In an *in vitro* co-culture system, CD25 expressing MHC-II⁺ ILC3 display a higher ability to bind IL-2 than auto-reactive T^H17 cells²⁴³, thereby limiting the availability of IL-2 to promote T^H17 cells survival. In this setting, addition of IL-2 reduced the apoptosis of T^H17, indicating that CD25⁺ MHC-II⁺ ILC3 induce the death of reactive T^H17 cells by depriving them of IL-2 (Fig.25).

Overall, CD4⁻ and CD4⁺ ILC3 also mediate mucosal inflammation and protection through their production of IL-17 and IL-22. This ILC3 lineage permits the establishment adaptive responses through the generation of SLOs, and controls the pool of autoreactive T^H17 cells, thereby preventing auto-immunity against harmless intestinal microbes.

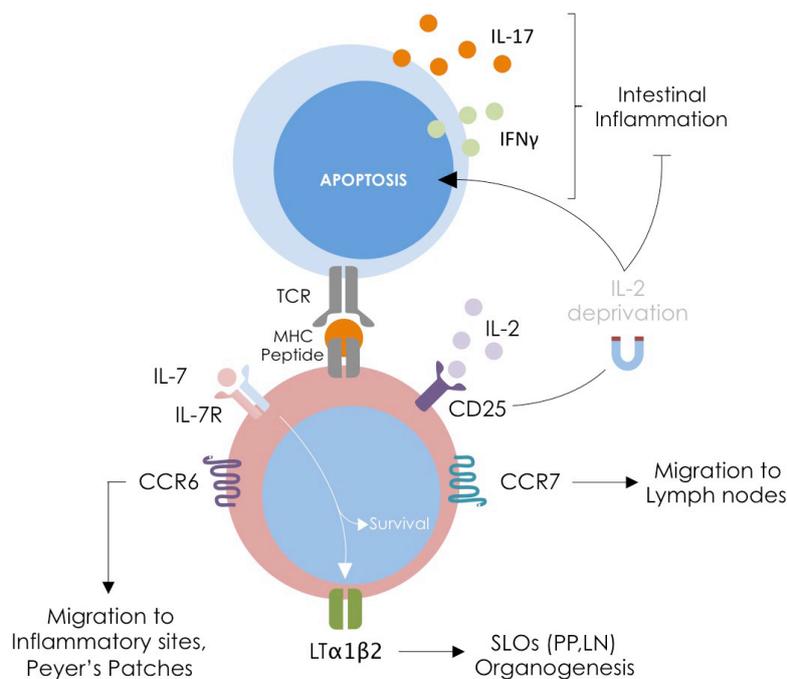


Fig 25 : Relative role of TGF β and Eomes in the establishment of ILC1a versus ILC1^b features

NKp46⁺ ILC3

NKp46⁺ ILC3 are comprised in the CD117^{int} CD117^{int} fraction¹²⁶ of ILC3, and appear to be restricted to the small intestine^{64, 123, 124, 178}. Unlike CD4⁺ ILC3, they are unable to produce IL-17 and are absent in murine fetus¹²⁶. NKp46 expression by ILC3 is detected in weanling mice (between 3 and 4 weeks of age), which coincide with food uptake and subsequent colonization by commensal microbes.

AhR exerts its transcriptional activity upon binding of cognate ligands, which includes phytochemicals such as flavonoids and glucosinolates that are found in conventional mouse diets. Weanling and adult mice with a targeted deletion of AhR in ROR γ t expressing cells display a reduced compartment of CD4⁻ ILC3ND, which includes NKp46⁺ ILC3, while CD4⁺ ILC3 were unaffected by Ahr loss¹⁹⁸.

A similar defect was observed in *Ahr* deficient mice, or mice fed with a diet exempt of phytochemical, indicating that nutrients-derived ligands drive the generation of adult CD4⁻ ILC3ND, but not fetal CD4⁺ ILC3, in an AhR-dependent manner. Moreover, a fraction of CD4⁻ ILC3ND compartment expressed the proliferation-associated marker Ki67, while the residuals CD4⁻ ILC3ND found in *Ahr* deficient mice were mostly Ki67⁻, implying that CD4⁻ ILC3ND contained cells that proliferated in an *Ahr* dependent manner¹⁹⁸.

Ahr transcripts are enriched in NKp46⁺ ILC3 when compared to CD4⁺ ILC3¹⁷⁸, indicating that AhR levels may influence NKp46⁺ ILC3 biology. NKp46⁺ ILC3 are mostly Ki67⁻, implying a loss of proliferative potential associated with maturation, and are virtually absent in *Ahr* deficient mice^{178, 197, 246}, which also lack CD4⁻ Ki67⁺ ILC3ND. Altogether, these results that CD4⁻ ILC3ND contained AhR dependent Ki67⁺ NKp46⁻ ILC3ND that arise post-natally, and suggest that this population potentially contain NKp46⁺ ILC3 precursors.

Tbx21 deficient mice completely lack NKp46⁺ ILC3^{56, 178}. In the small intestine, all CD127^{int} CD117^{int} ILC3 express T-bet, comprise both NKp46⁺ and NKp46⁻ cells, and lack CCR6 expression, the characterizing marker of lymphoid tissue inducing ILC3. Upon PMA-ionomycin stimulation, all T-bet⁺ ILC3 produce IL-22, IFN- γ , TNF α and GM-CSF, indicating that they share properties with both ILC1^a and ILC3.

Ahr deficient mice lack T-bet expressing ILC3, indicating that T-bet expression is probably downstream of AhR. Moreover, NKp46⁻ T-bet⁺ ILC3 exhibit are Ki67⁺, and emerge after birth. Finally, transfer of NKp46⁻ CCR6⁻ ILC3, which include a majority T-bet⁺ ILC3, in RAG^{KO/KO} *Il2rg*^{KO/KO} generated NKp46⁺ ILC3. As such, NKp46⁻ (T-bet⁺) ILC3 have been proposed to be the precursors for NKp46⁺ ILC3.

The factors that control NKp46⁺ expression ILC3 will be extensively discussed in the last part of this manuscript.

Still, the role of *Ncr1* expression in ILC3 remains elusive. Indeed, NKp46 crosslinking with platebound antibody fails to induce IL-22 production²⁴⁷, while mice deficient for *Ncr1*, or with conditional deletion of ROR γ t in NKp46 cells^{201, 248}, survive *Citrobacter rodentium* infection, indicating that neither NKp46 expression or NKp46⁺ ILC3 are required for IL-22 mediated intestinal defense against *Citrobacter rodentium*. As reports focused on the cytokine production in inflammatory models, the redundancy with other ILC3 or ILC1^a complicate the characterization of NKp46⁺ ILC3 biological function. As NKp46⁺ ILC3 are only found in the small intestine, NKp46⁺ ILC3 exert their function in close dependency with their environment, which in fine could be totally diverging from immune regulation.

As such, most of my work aimed to understand the biology of NKp46⁺ ILC3, and is presented in the Article N°2. *Ncr1* was a key marker for the identification of these IL-22 producing cells, although it was formerly recognized as an ILC1 defining marker. However, *Ncr1* expression is differentially regulated between ILC1 and ILC3, as NKp46⁺ ILC3 display lower amount of surface NKp46. Before addressing the function of NKp46 in ILC3, several questions had to be answered during my thesis, all of which focus on *Ncr1* expression in ILC3.

What is the dynamic of *Ncr1* expression in ILC3 ?

What specific features are associated with *Ncr1* expression in ILC3 ?

What external signals drive *Ncr1* expression in ILC3 ?

To answer these questions, I mainly relied on *Ncr1* fate-mapping mice, which allowed me to assess the kinetics of NKp46 expression through FACS analysis, as well as functional and phenotypic features. By setting up an in vitro culture system of isolated ILC3, I identified signals that controls *Ncr1* expression.



Article n°1

The intestinal mucosa is in close contact with commensal bacteria, which provides a unique stimulatory environment for immune cells. For instance, microbiota drives the generation of CX3CR1⁺ macrophages²⁴⁹, which samples luminal antigens via their transepithelial dendrites. CX3CR1⁺ establish residency throughout the small intestine lamina propria^{229, 250}, and are unable to migrate to lymph node to mediate antigen-presentation.

Still, mice deficient for CX3CR1 exhibit heightened susceptibility to DSS-induced colitis²⁵¹, a phenotype that is reminiscent of *Il22* deficient mice. In the small intestine, IL-22 promotes epithelial integrity, and induces the secretion of anti-microbial peptides, thereby tuning microbiota composition.

Group 3 innate lymphoid cells (ILC3) are the major source of IL-22, which secretion is triggered by myeloid-derived IL-23. CX3CR1⁺ macrophages are an important source of IL-23, but whether they can influence ILC3 homeostasis by other means has not been fully addressed.

In this report, we identify CX3CR1⁺ macrophages as a source of the chemokine CXCL16. Its cognate receptor, CXCR6, was indispensable for the localization of NKp46⁺ ILC3 in the lamina propria of the small intestine and the protection against the enteroinvasive pathogen *Citrobacter rodentium*. These results emphasize the importance of the CXCL16-CXCR6 axis in intestinal homeostasis, that is typified by the interplay between ILC3 and myeloid cells.

The Chemokine Receptor CXCR6 Controls the Functional Topography of Interleukin-22 Producing Intestinal Innate Lymphoid Cells

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<http://dx.doi.org/10.1016/j.immuni.2014.10.007>

SUMMARY

Interleukin-22 (IL-22) plays a critical role in mucosal defense, although the molecular mechanisms that ensure IL-22 tissue distribution remain poorly understood. We show that the CXCL16-CXCR6 chemokine-chemokine receptor axis regulated group 3 innate lymphoid cell (ILC3) diversity and function. CXCL16 was constitutively expressed by CX3CR1⁺ intestinal dendritic cells (DCs) and coexpressed with IL-23 after *Citrobacter rodentium* infection. Intestinal ILC3s expressed CXCR6 and its ablation generated a selective loss of the NKp46⁺ ILC3 subset, a depletion of intestinal IL-22, and the inability to control *C. rodentium* infection. CD4⁺ ILC3s were unaffected by CXCR6 deficiency and remained clustered within lymphoid follicles. In contrast, the lamina propria of *Cxcr6*^{-/-} mice was devoid of ILC3s. The loss of ILC3-dependent IL-22 epithelial stimulation reduced antimicrobial peptide expression that explained the sensitivity of *Cxcr6*^{-/-} mice to *C. rodentium*. Our results delineate a critical CXCL16-CXCR6 crosstalk that coordinates the intestinal topography of IL-22 secretion required for mucosal defense.

INTRODUCTION

Innate lymphoid cells (ILCs) comprise a related set of hematopoietic effector cells that participate in immune defense and tissue remodeling, especially at mucosal surfaces (reviewed in Spits et al., 2013). Three groups of ILC can be defined based on transcription factor requirements, cytokine production profiles, and roles in immunity (Spits and Di Santo, 2011). A first group of ILC (ILC1) includes natural killer cells (NK cells) and other interferon- γ (IFN- γ)-producing ILC that expresses the transcription factor T-bet (*Tbx21*) and play important roles in protection against viruses and intracellular pathogens (Bernink et al., 2013; Gordon et al., 2012; Klose et al., 2014; Townsend

et al., 2004). A second group of ILC (ILC2) includes “nuocytes” or natural helper cells that express the transcription factors Gata-3 and ROR α and produce interleukin-5 (IL-5) and IL-13 in response to parasitic infections, allergens, and viruses (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Wong et al., 2012). A third ILC group (ILC3) includes transcription factor ROR γ t⁺ lymphoid-tissue inducer cells (LTi cells) and natural cytotoxicity receptor (NCR)-expressing cells that have protective roles against extracellular bacteria and fungi and can be associated with organ-specific inflammation (Cella et al., 2009; Cupedo et al., 2009; Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008; Sawa et al., 2010). These different ILC groups have been identified in multiple tissues and in the circulation (Spits and Di Santo, 2011). In most organs, ILC subsets constitute a minor fraction of total lymphocytes; however, mucosal tissues represent the exception because these sites harbor multiple ILC subsets under steady-state conditions (Sonnenberg and Artis, 2012). The mechanisms that regulate ILC tissue distribution under normal conditions and during inflammation or infection are poorly understood.

IL-22 is a member of the IL-10 family that plays multiple roles in the regulation of various nonhematopoietic cells via the IL-22 receptor complex expressed on diverse epithelial cell types (enterocytes, goblet cells, Paneth cells) (reviewed in Sabat et al., 2014). These epithelial subsets have distinct anatomical functions and localizations within the intestinal mucosa. An effective IL-22-dependent epithelial response has critical consequences as illustrated by the absence of mucosal barrier containment and severe susceptibility of *Il22*^{-/-} mice to infection by *Citrobacter rodentium* or *Klebsiella pneumoniae* that can be reversed by exogenous administration of the antimicrobial peptide RegIII γ (Aujla et al., 2008; Zheng et al., 2008). This same mechanism might in part be involved in the anatomical restriction of lymphoid-resident commensal bacteria that can provoke systemic inflammation (Sonnenberg et al., 2012). A better understanding of the signals that regulate localized IL-22 production could offer a means to limit damage following infection and promote tissue remodeling and repair.

Cellular sources of IL-22 include a variety of innate and adaptive lymphocytes. In the case of *C. rodentium* infection,

ILC3-dependent production of IL-22 is critical for host survival and protects recombination-deficient mice lacking adaptive immunity (Zheng et al., 2008). Several groups have studied the localization of ROR γ t⁺ IL-22-producing ILC3 subsets that differentially express NKp46 and CD4. NKp46⁺ ILC3s are found in the intestinal lamina propria (LP) but are rare in cryptopatches; these cells lack CCR6 chemokine receptor expression and do not produce IL-17A (Satoh-Takayama et al., 2011; Satoh-Takayama et al., 2008; Sawa et al., 2010). In contrast, CD4⁺ ILC3 (LTi cells in fetal and adult) express CCR6, localize to cryptopatches and isolated lymphoid follicles, and produce IL-17A (Klose et al., 2013; Luci et al., 2009; Sawa et al., 2010). In addition, less well-defined CD4⁻NKp46⁻ ILC3 subsets have been identified that variably express T-bet and CCR6 (Buonocore et al., 2010; Klose et al., 2013; Sawa et al., 2010). These IL-22-producing ILC3 subsets share common features including a CD3⁻CD127⁺ phenotype and requirement for ROR γ t expression in order to develop and produce IL-22 (reviewed in (Spits et al., 2013)). The identification of phenotypically and functionally distinct IL-22-producing ILC3 subsets with different tissue localization within mucosal surfaces raises questions about the mechanisms that control ILC3 localization within tissues and the importance of directing IL-22 to discrete epithelial compartments.

Mononuclear phagocytes (MPs), comprising monocytes, dendritic cells (DCs), and macrophages are central coordinators of adaptive immunity but also “crosstalk” with innate lymphocytes (such as NK cells) in order to regulate their maturation and function. Subsets of functionally distinct intestinal lamina propria macrophages and DC (CD11c^{hi}, MHC class II^{hi} cells) have been identified based on expression of CD103, CD11b, and CX3CR1 (Varol et al., 2010). CD103⁺CD11b⁺ DCs represent a major population in the lamina propria that is an important producer of IL-23 in response to bacterial PAMPs (Kinnebrew et al., 2012). In contrast, CD103⁺CD11b⁻ lamina propria DCs are potent inducers of regulatory T cells through a transforming growth factor β and retinoic acid-dependent mechanism (Coombes et al., 2007). Lastly, a subset of CD11c^{hi}MHC class II^{hi} cells in the lamina propria has the CD103⁻CD11b⁺ phenotype with intermediate CX3CR1 expression (Cerovic et al., 2013). These CX3CR1⁺ MPs express macrophage markers (F4/80, CD64) and have high phagocytic activity but also show the ability to form transepithelial dendrites (Chieppa et al., 2006; Niess et al., 2005). Because intestinal macrophages and/or DCs are potential sources of IL-23 that can regulate IL-22 production during infection (Kinnebrew et al., 2012; Satpathy et al., 2013), the regulated interactions of DCs, macrophages, and MPs with diverse ILC subsets may represent an important mechanism to maintain gut homeostasis. Lymphotoxins play an important role in ILC regulation via intestinal DCs during *C. rodentium* infection (Tumanov et al., 2011), and recent reports implicate CX3CR1⁺ hematopoietic cells in the regulation of IL-22 production in this context (Longman et al., 2014; Manta et al., 2013). In contrast, little is known about the mechanisms that orchestrate intestinal IL-22 topography.

Here we investigate the importance of the CXCL16-CXCR6 chemokine-chemokine receptor axis between CX3CR1⁺ DC and ILC3 subsets in the orchestration of tissue-specific topography of IL-22 secretion in the intestine under steady-state conditions and in response to mucosal pathogens.

RESULTS

CX3CR1 Expression Is Required for Homeostasis of IL-22-Producing ILC3

Our understanding of the signals that regulate ILC migration within tissues is in its infancy. We analyzed whether myeloid cells deliver chemokine signals that influence ILC3 positioning and regulate steady-state ILC3 homeostasis. Resident macrophages and DCs in the gut differentially express the chemokine receptor CX3CR1 (that binds fractalkine) and chemokine signaling through CX3CR1 controls IL-22 production during *C. rodentium* infection (Longman et al., 2014; Manta et al., 2013). In order to study the influence of CX3CR1 expressing cells on intestinal ILC3 homeostasis and function, we analyzed CX3CR1-deficient mice (Jung et al., 2000). Frequencies and absolute numbers of ILC3 (CD3⁻CD127⁺ROR γ t⁺ cells) were reduced in *Cx3cr1*^{gfp/gfp} mice compared to controls that could be accounted for by a significant 60% reduction in the absolute numbers of NKp46⁺ ILC3 (Figures 1A and 1B). In contrast, steady-state numbers of CD4⁺ and CD4⁻NKp46⁻ (double-negative or “DN”) ILC3, ILC2 (CD3⁻CD127⁺ROR γ t⁻Gata3^{hi}), and NK1.1⁺ NK cells were not changed (Figures 1A and 1B; data not shown).

We next examined the influence of CX3CR1 deficiency on cytokine production by ILC3 subsets. Because IL-23 production from intestinal CD11b⁺ DCs can stimulate ILC3-dependent IL-22 production (Cox et al., 2012; Longman et al., 2014), we assessed both constitutive and IL-23-induced IL-22 production from ILC3 subsets in vitro. Comparing *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice, we found that while percentages of the different ILC3 subsets that constitutively produced IL-22 were decreased (Figure 1C), the absolute numbers of IL-22-producing NKp46⁺ ILC3s were reduced in the absence of CX3CR1 while CD4⁺ and DN ILC3 were unaffected (Figure 1D). When stimulated ex vivo with IL-23, these differences between *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice were more obvious with an 80% decrease in the number of NKp46⁺ ILC3 that could produce IL-22 (Figures 1E and 1F). A trend toward lower numbers of IL-22⁺ DN ILC3 was also observed although this was not significant. Since ILC3 subsets fail to express CX3CR1 (see Figure S1A available online), these results suggest that CX3CR1 expression is required for steady-state and IL-23-inducible IL-22 production from diverse ILC3 subsets. The reduced IL-23 responsiveness of ILC3 subsets was not due to changes in *Il23r* expression (Figure S1B).

Intestinal Chemokine CXCL16 Expression Is Regulated by CX3CR1

Given the effects of CX3CR1 ablation on the homeostasis of IL-22-producing intestinal ILC3, we hypothesized that CX3CR1⁺ myeloid cells might maintain ILC3 through elaboration of soluble factors. We analyzed chemokine expression in isolated CD11b⁺ and CD11b⁻ intestinal DC subsets (MHC II⁺CD11c^{hi} cells) using targeted microarrays. While CCL19 and CCL20 were expressed at low amounts, we documented a strong expression of CXCL16 by CD11b⁺ DC and clear CXCL16 expression in CD11b⁻ DC (Figure 2A). Moreover, we found that CX3CR1 was critical for *Cxcl16* expression by CD11b⁺ DCs (Figure 2B) and further identified a role for CX3CR1 in regulating *Il23a* expression in intestinal DCs (Figure 2B), confirming a recent report (Longman et al.,

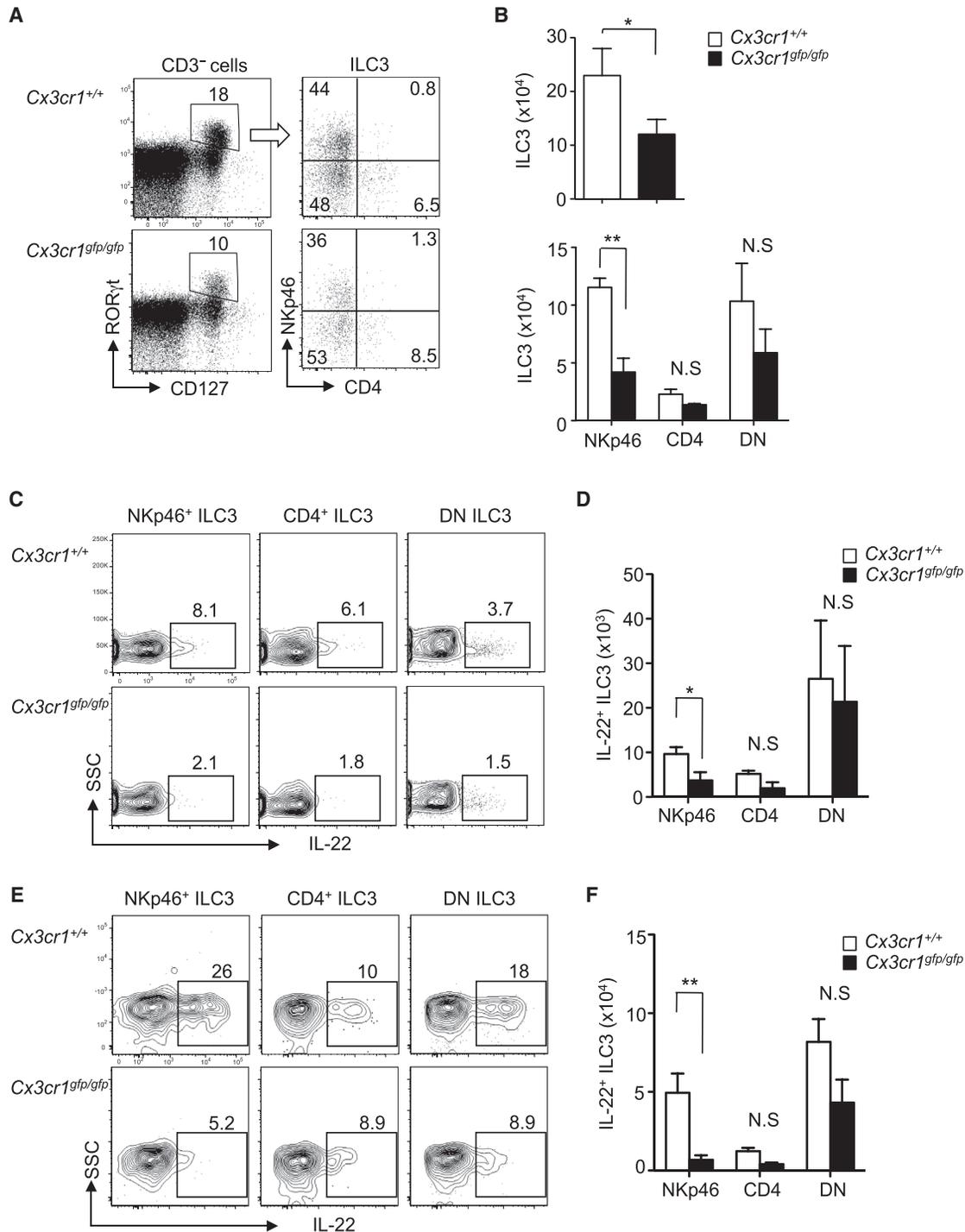


Figure 1. Characterization of IL-22 Producing ILC3 Subsets in Small Intestine from *Cx3cr1* Reporter Mice

(A) Analysis of ILC3s (CD3⁻CD127⁺RORγt⁺ cells) in small intestine lamina propria lymphocytes of *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice. Numbers indicate percent of gated CD3⁻CD127⁺RORγt⁺ cells (left panels) and CD4 and NKp46 expressing ILC3 subsets (right panels).

(B) Absolute numbers of total ILC3, NKp46⁺ ILC3, CD4⁺ ILC3, and CD4⁻ NKp46⁻ (DN) ILC3s from SI LPLs of *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice.

(C and E) Analysis of IL-22 production by ILC3 subsets in *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice. Boxes indicated percentages of positive cells. SI LPLs were stimulated without (C) or with (E) IL-23 in vitro for 4 hr.

(D and F) Absolute numbers of IL-22-producing ILC3 subsets from *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice stimulated without (D) or with (F) IL-23. Data are representative of three independent experiments with n = 4–6 mice analyzed for each genotype. Data shown are mean values ± SEM. *, p value < 0.05. **p < 0.01. See also Figure S1.

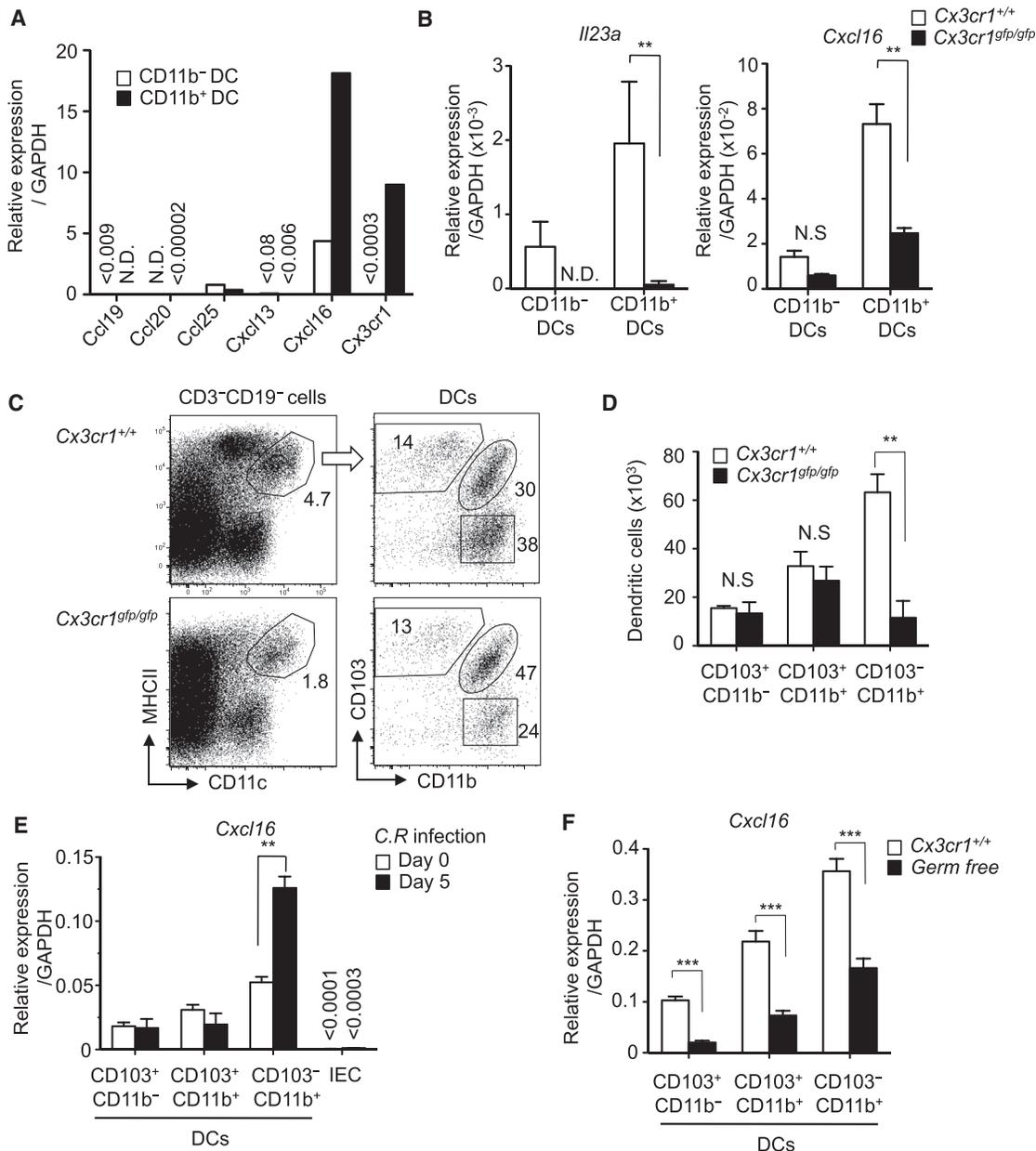


Figure 2. The Chemokine CXCL16 Is Expressed by Intestinal CD11b⁺ cDCs

(A) Analysis of chemokine expression by subsets of intestinal MHC II⁺CD11c⁺ DCs from *Rag2*^{-/-} mice.

(B) *Il23a* (left) and *Cxcl16* (right) expression in intestinal MHC II⁺CD11c⁺ DC subsets from *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice. Three mice of each genotype were analyzed.

(C) CD11b and CD103 expression on intestinal MHC II⁺CD11c⁺ DC cells from *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice. Data are representative of three independent experiments with four to six mice of each genotype analyzed.

(D) Absolute numbers of intestinal MHC II⁺CD11c⁺ DC subsets in *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/gfp} mice.

(E) *Cxcl16* expression in isolated intestinal MHC II⁺CD11c⁺ DC subsets from *Cx3cr1*^{+/+} mice prior to (open bars) or 5 days postinfection with *C. rodentium* (filled bars). *Cxcl16* expression in IECs is also shown. Three mice of each genotype were analyzed.

(F) *Cxcl16* expression in isolated intestinal MHC II⁺CD11c⁺ DC subsets from *Cx3cr1*^{+/+} (open bars) or germ-free mice (filled bars). Four mice of each genotype were analyzed. Data shown are mean values \pm SEM. **p value < 0.01. ***p value < 0.001. See also Figure S2.

2014). Subsets of intestinal DCs (MHC II⁺CD11c^{hi} cells) have been identified based on the CD103 and CD11b expression (Varol et al., 2010). CX3CR1 expression by intestinal MHC II⁺CD11c^{hi} cells was restricted to CD103⁻CD11b⁺ cells (Varol et al., 2010; Figure S2A). CX3CR1 deficiency clearly affected in-

testinal myeloid cell homeostasis with a marked reduction in absolute numbers of CD103⁻CD11b⁺ intestinal DCs (Figures 2C and 2D). Steady-state *Cxcl16* expression was highest in CD103⁻CD11b⁺ DCs, and *Cxcl16* expression was strongly induced in this subset in response to infection by the intestinal

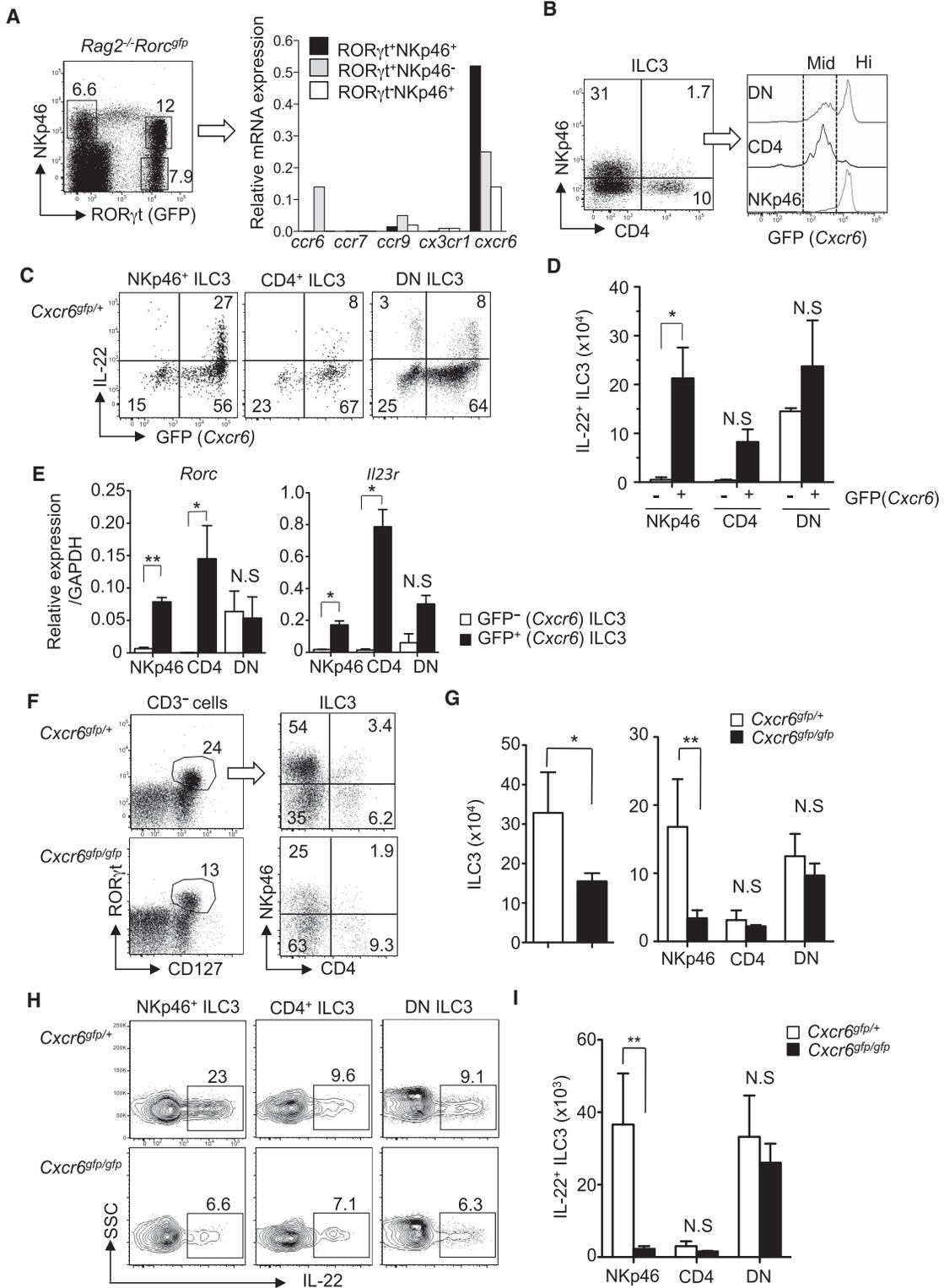


Figure 3. CXCR6 Is Essential for Homeostasis of Gut IL-22⁺NKp46⁺ ILC3s

(A) Analysis of chemokine receptor expression in sorted ILC cell subsets from SI LPLs of *Rag2^{-/-}Rorc^{GFP}* mice. (B) *Cxcr6* expression (GFP) in ILC3 subsets (NKp46, CD4, and DN) from the SI LPLs of *Cxcr6^{GFP/+}* mice. (C) IL-22 expression in ILC3 subsets from *Cxcr6^{GFP/+}* mice. IL-22 versus *Cxcr6* expression (GFP) is shown in IL-23 stimulated NKp46, CD4, and DN ILC3 subsets. (D) Absolute numbers of IL-22⁺ NKp46, CD4, or DN ILC3 from SI LPLs of *Cxcr6^{GFP/+}* mice. Black bars indicate *Cxcr6* expressing cells.

(legend continued on next page)

pathogen *C. rodentium* (Figure 2E). Previous studies have shown that MHC II⁺CD11c^{mid} cells, including intestinal monocytes and macrophages, are also reduced in *Cx3cr1^{gfp/gfp}* mice (Yu et al., 2007), a result that we could confirm (Figure S2B). However, intestinal monocytes failed to express significant amounts of *Cxcl16*, in contrast to CD11c^{hi} DCs (Figure S2C). Intestinal endothelial cells expressed low amounts of *Cxcl16* that was regulated in a CX3CR1-dependent fashion (Figure S2D). CD64⁺ macrophages and Ly6C⁺ inflammatory monocytes also expressed markedly lower *Cxcl16* amounts compared with CD103⁻CD11b⁺ intestinal DCs that were not significantly modified after *C. rodentium* infection (Figures S2E–S2G).

To gain an insight into the mechanisms regulating *Cxcl16* expression in CD11b⁺ intestinal DCs, we analyzed germ-free C57BL/6 mice. We found that gut CD11b⁺ cells had reduced *Cxcl16* amounts in the absence of commensal flora suggesting that microbial stimuli regulate chemokine expression (Figure 2F). Together, these results link *Cxcl16* expression in intestinal CX3CR1⁺ DCs with steady-state homeostasis and IL-23-inducible IL-22 production by gut ILC3.

The Chemokine Receptor CXCR6 Is Required for Homeostasis of Intestinal ILC22 Subsets

Since CX3CR1 deficiency strongly affected CXCL16 expression by intestinal myeloid cells, we next assessed how lack of CXCL16 function might impact on intestinal ILC3 subset homeostasis and function. We therefore screened gut ILC3 subsets (NKp46⁺, CD4⁺, DN) for expression of chemokine receptors (Figure 3A). *Ccr6*, *Ccr7*, and *Ccr9* were expressed in low amounts by ILC3 subsets, while *Cxcr6* was highly expressed, especially in NKp46⁺ ILC3s (Figure 3A).

We confirmed these results using mice bearing a targeted insertion of a GFP-reporter into the *Cxcr6* locus (*Cxcr6^{gfp/+}* mice) (Geissmann et al., 2005). ILC subsets differentially expressing NKp46 and CD4 transcribed the *Cxcr6* locus with the vast majority of NKp46⁺ and CD4⁺ cells expressing CXCR6, while the DN cells harbored both CXCR6⁺ and CXCR6^{mid} cells (Figure 3B). GFP expression was highest in NKp46⁺ ILC3s and on a subset of DN ILC3s and lower on CD4⁺ ILC3s (Figure 3B) suggesting that CXCR6 expression could be correlated with differential homing and/or function of ILC3 subsets. Along these lines, IL-23-stimulated IL-22 production from both NKp46⁺ and CD4⁺ ILCs was clearly correlated with CXCR6 expression (Figure 3C). IL-22⁺ cells were restricted to CXCR6^{hi} cells, whereas IL-22⁺ cells in the DN subset were either CXCR6^{hi} or CXCR6⁻ (Figure 3D). To gain insight into the mechanisms that could explain the differential IL-22 expression by CXCR6 subsets, we performed molecular analysis of ILC3 subsets that were GFP⁺ or GFP⁻. *Rorc* expression was enriched in GFP⁺ ILC3s that expressed NKp46 or CD4 and was detected in both GFP⁺ and GFP⁻ DN ILC3s (Figure 3E). Moreover, *Il23r* expression was

also strongly correlated with GFP expression (Figure 3E) suggesting that CXCR6-expressing ILC3s are the major responders to IL-23 during inflammation and infection. Incubation of lamina propria (LP) cells from *Cxcr6^{gfp/+}* mice with CXCL16 did not alter the proportions of GFP⁺ ILC3s and addition of CXCL16 to IL-23-stimulated cultures did not affect the percentages of IL-22-producing cells (Figure S3A), suggesting that CXCL16 does not act as a maturation or costimulatory factor for intestinal ILC3s.

To assess the effect of CXCR6 deficiency on ILC subset distribution, we analyzed *Cxcr6^{gfp/gfp}* mice as the GFP reporter insertion simultaneously inactivates the corresponding *Cxcr6* allele (Geissmann et al., 2005). CXCR6 deficiency does not affect RORγ⁺ ILC differentiation during embryogenesis (Possot et al., 2011) and *Cxcr6^{gfp/gfp}* mice demonstrated normal development of Peyer's patches and lymph nodes (data not shown). Absolute numbers of ILC3s were reduced by *Cxcr6* ablation (Figures 3F and 3G), and moreover, the ILC3 subset distribution was altered, with a clear reduction in the proportions and absolute numbers of NKp46⁺ cells (Figure 3G).

Intestinal ILC3s from mice deficient in *Cxcr6* exhibited reduced IL-22 production ex vivo. IL-23-stimulated IL-22 production from NKp46⁺ cells was strongly reduced, whereas IL-22 production from CD4⁺ cells and DN cells was not significantly modified (Figure 3H). When calculating absolute numbers of IL-22⁺ cells, the selective negative effect of *Cxcr6* deletion against NKp46⁺ IL-22⁺ cells was clear: over 95% of these cells were lost, in contrast with numbers of IL-22⁺ CD4⁺ and DN cells that remained essentially unchanged in *Cxcr6^{gfp/gfp}* mice (Figure 3I). Together, these results demonstrate that CXCR6 expression marks IL-23 responsive IL-22-producing ILC3 subsets and that CXCR6 plays a critical role in the homeostasis of intestinal NKp46⁺ ILC3s.

We also analyzed the effects of *Cxcr6* deletion on intestinal CD4⁺ T cells. We found no differences in the absolute numbers of gut CD4⁺ T cells in *Cxcr6^{gfp/+}* versus *Cxcr6^{gfp/gfp}* mice (Figure S3B). About 18% of intestinal CD4⁺ T cells expressed GFP⁺ and there was no reduction in IL-22⁺ T cells in the absence of CXCR6 (Figures S3C and S3D).

Selective Loss of IL-22-Dependent Effectors in the Villus Epithelium of *Cxcr6^{gfp/gfp}* Mice

We next assessed the impact of CXCR6 deficiency on the tissue localization of RORγ⁺ ILC in the small intestine. Previous studies have localized NKp46⁺RORγ⁺ ILC to the intestinal villus, whereas CD4⁺RORγ⁺ and DN RORγ⁺ ILCs are clustered in cryptopatches and isolated lymphoid follicles (ILF) (Luci et al., 2009; Satoh-Takayama et al., 2011; Satoh-Takayama et al., 2008; Sawa et al., 2010). This dichotomy of ILC3 positioning suggests different mechanisms for anatomical partitioning within the intestine. Using *Cxcr6^{gfp/+}* mice, we found that CD3⁻CXCR6⁺RORγ⁺ ILCs were distributed in anatomically

(E) Analysis of *Rorc* and *Il23r* expression in sorted *Cxcr6⁻* (GFP⁻) or *Cxcr6⁺* (GFP⁺) ILC subsets from *Rag2^{-/-}Cxcr6^{gfp/+}* mice.

(F) Analysis of ILC3 subsets in SI LPLs from *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice. Numbers indicate percent of gated CD3⁻CD127⁺RORγ⁺ cells (left panels) and CD4⁺ and NKp46-expressing ILC3 subsets (right panels).

(G) Absolute numbers of total ILC3, NKp46⁺, CD4⁺, and DN ILC3 subsets from SI LPLs of *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice.

(H) Analysis of IL-22 expression in intestinal ILC subsets from *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice. SI LPLs were stimulated with IL-23 for 4 hr in vitro.

(I) Absolute numbers of IL-22 producing ILC3 subsets in *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice. Data are representative of >3 experiments with n = 4–6 mice analyzed for each genotype. *p value < 0.05. **p < 0.01. See also Figure S3.

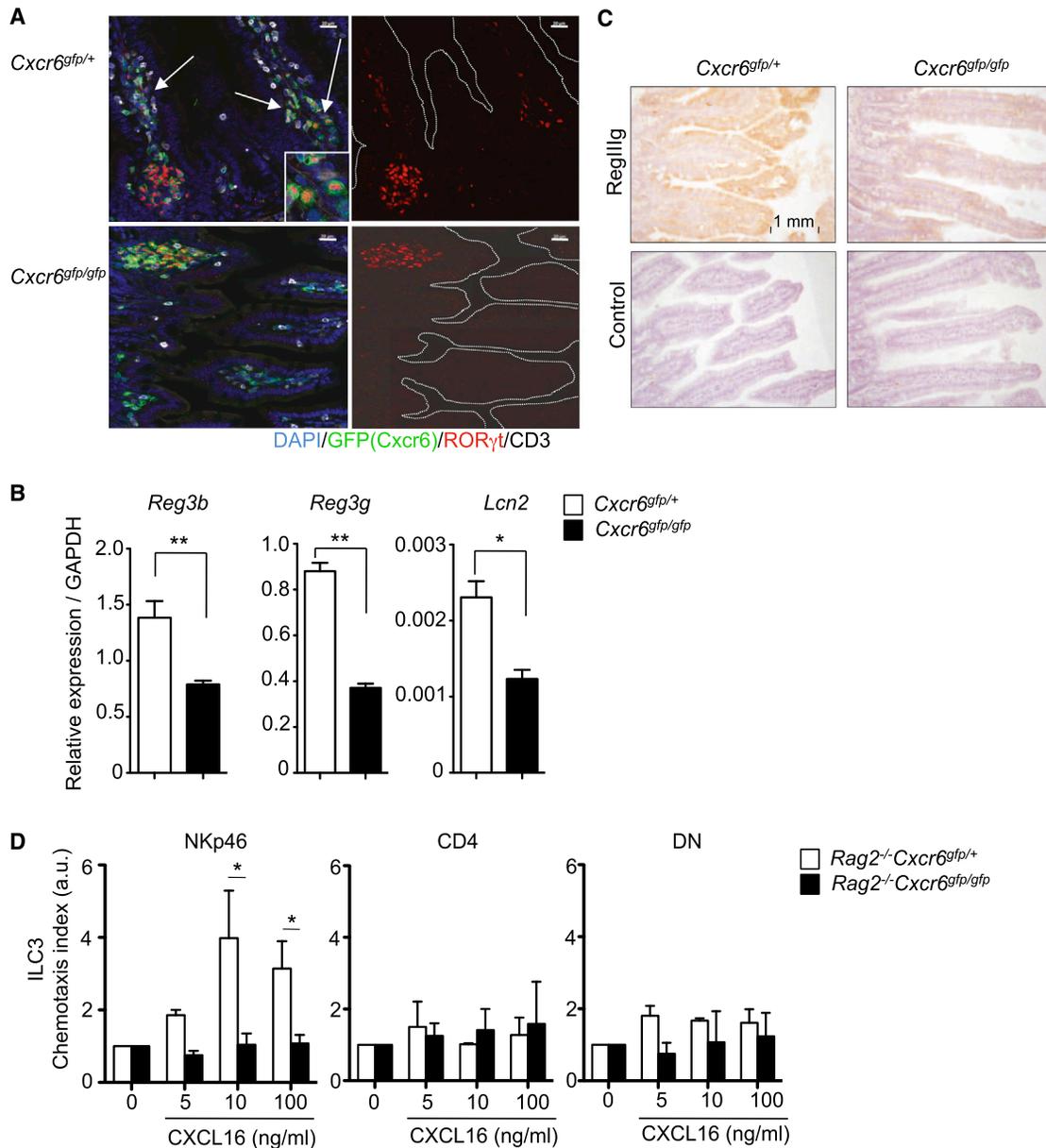


Figure 4. Loss of IL-22-Mediated Responses in the Villus Epithelium of *Cxcr6^{gfp/gfp}* Mice

(A) Immunohistological analysis of CXCR6⁺ ILC3 in small intestine from *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice. Sections were stained with DAPI (blue), anti-GFP (CXCR6; green), anti-ROR γ t (red), and anti-CD3 (white). Scale bar represents 20 μ m. Original magnification: $\times 20$. Representative results in one of three independent experiments are shown.

(B) Analysis of *Reg3g*, *Reg3b*, and *Lcn2* expression by SI LPLs and intestinal epithelial cells (IEC) isolated from *Rag2^{-/-}Cxcr6^{gfp/+}* and *Rag2^{-/-}Cxcr6^{gfp/gfp}* mice ($n = 5$ mice of each genotype). Results are normalized to *Gapdh*. * p value < 0.05.

(C) Immunohistochemistry of villus epithelium (small intestine) from *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice stained with/without RegIII γ -specific antibodies and counterstained with hematoxylin. Scale bar represents 1 mm.

(D) Transwell migration assay using SI LPLs by *Rag2^{-/-}Cxcr6^{gfp/+}* and *Rag2^{-/-}Cxcr6^{gfp/gfp}* mice. Cells were cultured with or without ligand *Cxcl16* (5, 10, and 100 ng/ml). For analysis, cells were gated on ILC3 (CD3⁻CD127⁺ROR γ t⁺) cells. Data are compiled from six independent experiments. * p value < 0.05. ** p < 0.01. See also Figure S4.

distinct regions (Figure 4A). Clusters of ROR γ t⁺ ILCs were found in patches at the base of the intestinal villi that resembled cryptopatches or ILF. These include CD4⁺ LTi-like cells that express CCR6 (Klose et al., 2013; Sawa et al., 2010); a fraction of these ROR γ t⁺ ILCs coexpressed CXCR6. A second subset of

ROR γ t⁺CXCR6⁺ ILCs was found interspersed throughout the LP (Figure 4A) that includes NKp46⁺ ILC3 (Sato-Takayama et al., 2008). Intestinal CXCR6⁺ T cells could also be detected in the LP where a fraction expressed ROR γ t (Figure 4A). In *Cxcr6^{gfp/gfp}* mice, clusters of ROR γ t⁺ ILC were retained;

however, a striking loss of ROR γ t⁺ ILCs was evident in the LP (Figure 4A). Some GFP⁺ T cells remained in the villi, although these also lacked detectable ROR γ t expression. These results demonstrate that CXCR6 expression is essential for homeostasis of ROR γ t⁺ ILCs in the villus LP, but not for ILC3 in cryptopatches or ILF.

Expression of antimicrobial peptides *Reg3b*, *Reg3g*, and *Lcn2* in intestinal epithelial cells (IECs) was strongly reduced in the absence of CXCR6 (Figure 4B). Immunohistological analysis of RegIII γ protein revealed obvious epithelial cell staining throughout the intestinal villus in *Cxcr6*^{gfp/+} mice whereas this staining was markedly reduced in *Cxcr6*^{gfp/gfp} mice (Figure 4C). These results demonstrate that ROR γ t⁺ ILCs and T cells in the LP are critical for maintaining IL-22-dependent effector proteins required for barrier protection. Moreover, these results suggest that ROR γ t⁺ innate and adaptive lymphocytes use distinct mechanisms for their homeostasis in the villus versus cryptopatch and ILF regions.

Despite their absence from the intestinal villus, absolute numbers of intestinal ROR γ t⁺ T cells were not altered in the absence of CXCR6 (Figure S4A). We next assessed whether the absence of villus ROR γ t⁺ ILCs and T cells in CXCR6 deficiency were linked. We analyzed ILC3 distribution in *Rag2*^{-/-} mice that were either CXCR6-sufficient (*Rag2*^{-/-}*Cxcr6*^{gfp/+}) or -deficient (*Rag2*^{-/-}*Cxcr6*^{gfp/gfp}). We found that CXCR6⁺ ILC3s normally localized within the villus in the absence of T cells but that ILC3s were missing from *Rag2*^{-/-} mice lacking CXCR6 (Figure S4B). This result suggests that CXCR6 was directly required to localize ILC3 within the intestinal lamina propria.

We next assessed the migratory capacities of ILC3 subsets from *Cxcr6*^{gfp/+} mice to CXCL16 using a transwell assay. We found that NKp46⁺ ILC3s selectively migrated in a dose-dependent fashion to CXCL16, whereas CD4⁺ and DN subsets did not (Figure 4D). As expected, migrating NKp46⁺ ILC3 were GFP⁺ (Figure S4C). These results demonstrate that NKp46⁺ ILC3 express functional CXCR6 that allows their migration toward CXCL16 and that CXCR6 is required for normal anatomical localization of NKp46⁺ ILC3 within the intestinal lamina propria. While DN ILC3s do not migrate to CXCL16, this ligand might trigger other functions within this ILC3 subset.

CXCR6 Is Required for IL-22-Dependent Mucosal Defense against Bacterial Infection

Since defense against *C. rodentium* induces high amounts of IL-22 that are critical for host survival (Zheng et al., 2008), this model provides a means to assess the role for innate and adaptive IL-22 sources for immunity against intestinal pathogens. When analyzing the early immune response to *C. rodentium* (day 5 postinfection), we found that intestinal NKp46⁺, and to a lesser extent “DN” ILC3s, were the predominant innate source of intestinal IL-22 (Figures S5A–S5D). To assess the role for CXCR6⁺ ILC3s in *C. rodentium* defense, we compared the response of *Rag2*^{-/-}*Cxcr6*^{gfp/+} and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice to this enteric pathogen. In *Cxcr6*^{gfp/+} mice, IL-22 production was induced in all ILC3 subsets (NKp46, CD4, “DN”) in response to *C. rodentium* and IL-22 expression was correlated with CXCR6 expression (Figure 5A; note that lamina propria lymphocytes (LPLs) were not restimulated with IL-23 in vitro). As was the case in Rag-competent hosts (Figures S5A–S5D), the increase in

absolute numbers of IL-22-producing cells was largely due to NKp46⁺ ILC3s, whereas numbers of IL-22⁺ CD4 remained stable and “DN” cells were slightly increased in this model of “innate-only” immunity (Figure 5B, left panel). In contrast, the absolute numbers of IL-22⁺ ILC3s did not increase in *C. rodentium*-infected mice lacking CXCR6 expression (Figure 5B, right panel).

As loss of CXCR6 selectively ablated ILC3s in the villus lamina propria in both Rag-competent and Rag-deficient mice (Figure 4A; Figure S4B), we assessed whether residual IL-22-production would be sufficient to protect the host against infection by *C. rodentium*. *Rag2*^{-/-}*Cxcr6*^{gfp/+} and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice were gavaged with bioluminescent *C. rodentium* (Wiles et al., 2006) and survival was assessed. We also noninvasively monitored *C. rodentium* growth during the infection (Serafini et al., 2014). As expected (Satoh-Takayama et al., 2008; Zheng et al., 2008), control *Rag2*^{-/-} and *Rag2*^{-/-}*Cxcr6*^{gfp/+} mice resisted infection up to 40 days; in contrast, *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice were highly susceptible and succumbed to infection within 2 weeks (Figure 5C). Accordingly, *C. rodentium* growth was controlled in CXCR6-competent *Rag2*^{-/-} mice, whereas infection spread in *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice (Figures 5D and 5E). Similar results were obtained in Rag-competent hosts lacking *Cxcr6* (Figures S5E and S5F). During *C. rodentium* infection, inducible IL-22 triggers production of antimicrobial peptides from intestinal epithelial cells (Zheng et al., 2008). We found that intestinal epithelial cell (IEC) expression of *Reg3b* and *Reg3g* were clearly reduced in *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice compared to *Rag2*^{-/-}*Cxcr6*^{gfp/+} mice at day 5 postinfection (Figure 5F) and IL-22 secretion from small intestine LP cells was reduced in *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice (Figure 5G). Moreover, administration of recombinant IL-22 to *C. rodentium*-infected *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice or *Cxcr6*^{gfp/gfp} mice could protect against early demise (Figure 5H; Figure S5G), strongly implicating defective IL-22 production in the heightened susceptibility of these mice to this pathogen. Taken together, our results define a coordinated crosstalk between CX3CR1⁺ DC and ILC3 subsets that orchestrates IL-22 production within discrete regions of the intestinal epithelium. CXCL16/CXCR6 interactions represent a critical mechanism to ensure mucosal barrier function under steady-state conditions and in response to infection.

DISCUSSION

A diverse set of ILCs regulates immune responses to pathogens at mucosal barriers (reviewed in Spits et al., 2013; Spits and Di Santo, 2011). While the mechanisms that control the effector functions of distinct ILC subsets are beginning to be unraveled, our understanding of the mechanisms that control ILC localization within different organs and tissues remain limited (Walker et al., 2013). Moreover, the functional consequences of distinct ILC distribution patterns are not understood. Our results shed light on these questions by demonstrating the critical requirement for CXCR6 in the homeostasis and localization of IL-22 producing cells within the intestinal LP, especially for NKp46⁺ ILC3s. The increased susceptibility of *Cxcr6*^{gfp/gfp} mice to *C. rodentium* infection highlights the importance of CXCL16-mediated tissue distribution of IL-22 producing subsets that are required for early host defense against pathogenic microbes.

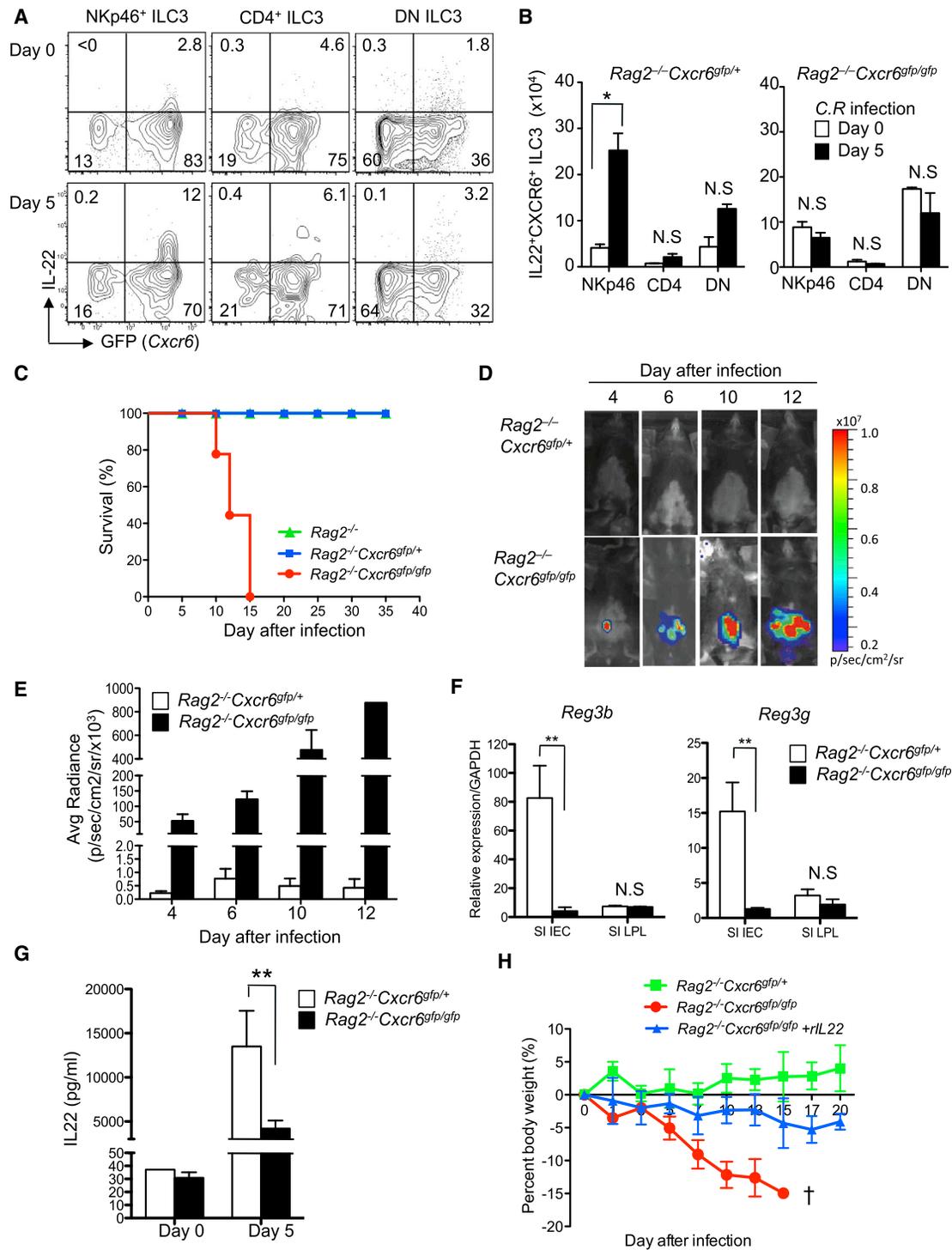


Figure 5. CXCR6 Is Required for ILC3-Mediated Mucosal Defense against Bacterial Infection

(A) Analysis of IL-22⁺ ILC3 in SI LPLs following *Citrobacter rodentium* infection. *Rag2*^{-/-}*Cxcr6*^{gfp/+} mice were orally gavaged with 1 × 10⁹ bacteria. Numbers indicate percent positive cells ILC3 subsets. Representative results from four independent experiments.

(B) Absolute numbers of IL-22 producing ILC (CD127⁺CD3⁺) in SI LPLs of *Rag2*^{-/-}*Cxcr6*^{gfp/+} (left panel) and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice (right panel) at steady-state (open bars) or following *C. rodentium* infection (day 5 postinfection; black bars). SI LPLs were cultured for 4 hr without stimulation in the presence of GolgiPlug. Four mice of each genotype were analyzed in two independent experiments.

(C) Survival after *C. rodentium* infection in *Rag2*^{-/-}, *Rag2*^{-/-}*Cxcr6*^{gfp/+}, and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice. Mice were orally gavaged with 1 × 10⁹ bacteria. Data are representative of four independent experiments (n = 6 for *Rag2*^{-/-} and n = 9 for *Rag2*^{-/-}*Cxcr6*^{gfp/+} mice, n = 10 for *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice).

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Previous studies used gene profiling to identify several chemokine receptors that are expressed by NKp46⁺ cells and CD4⁺ ROR γ t⁺ ILC3 subsets (Cella et al., 2009; Luci et al., 2009). However, our understanding of the functional importance of these receptors for ILC3 homeostasis and function is not complete. CXCR5 ablation has been shown to affect LTi function, with defects in lymph nodes and Peyer's patches development, while CCR7 expression on LTi cells has been suggested to reinforce lymphoid tissue organogenesis through reciprocal activation by mesenchymal stromal cells that express CCL19 (Ohl et al., 2003). CCR6 is expressed by CD4⁺ ILC3 and by a subset of DN ILC3s (Klose et al., 2013; Sawa et al., 2010). Although the absence of CCR6 does not affect LN or Peyer's patch formation, CCR6 ligands are involved in cryptopatch and ILF maturation in the intestine that play a direct role in regulating commensal communities (Bouskra et al., 2008). In contrast, CCR6⁻ NKp46⁺ ILC3s develop independently of this receptor (Satoh-Takayama et al., 2008; Sawa et al., 2010).

A previous report identified CXCR6 as a marker of ROR γ t⁺ precursors in the fetal liver and spleen (Possot et al., 2011). While the absence of CXCR6 did not affect development of fetal ROR γ t⁺ cells, this receptor has a critical role in homeostasis of the NKp46⁺ ILC3 subset in adult mice. The age-dependent effect of CXCR6 may be explained by chemokine receptor redundancy; ROR γ t⁺ fetal lymphoid precursors were shown to express CXCR5, CCR6, and CCR7 (Possot et al., 2011). In contrast, CXCR6 might have its main role in the development of certain ILC subsets that appear after birth, such as NKp46⁺ ILC3s (Sawa et al., 2010).

We found that CXCR6 expression marked a functionally mature subset within intestinal ILCs. IL-22 production was correlated with *Cxcr6* expression in NKp46⁺, CD4⁺, and a subset of DN ILC3s. These CXCR6⁺ cells had higher expression of *Rorc* and *I23r* that could explain their heightened capacity to produce IL-22. The molecular mechanisms that control chemokine receptor expression in ILCs are not well defined. Previous studies have shown that CCR6 and IL-23R are ROR γ t-dependent target genes (Yamazaki et al., 2008), and we found that *Cxcr6* expression correlated with *Rorc* in NKp46⁺ and CD4⁺ ILC3 subsets. As such, *Rorc* might program a constellation of surface receptors that allow ILC3 subsets to distribute within the intestine, ready to receive the relevant soluble signals from sentinel cells for their activation. Still, additional transcriptional regulators must be operating because ILC3 subsets differentially express CCR6 and CXCR6 that apparently allows them to home to distinct gut microenvironments.

Whatever the mechanism, the ability of CXCR6-expressing ILCs to produce IL-22 would ultimately target this factor to the corresponding CXCL16-rich regions within the intestine. The

transmembrane-bound CXCL16 is expressed in many cell types (Matloubian et al., 2000), including both hematopoietic (macrophages) and nonhematopoietic (epithelial, endothelial) lineages that vary during ontogeny. We found that CD11b⁺ intestinal DCs were the major *Cxcl16* expressing cells in the adult under steady-state conditions. In addition, *Cxcl16* expression in these cells increased following intestinal infection, in agreement with previous studies (Tohyama et al., 2007). As CD11b⁺ inflammatory phagocytes also express *I23a* under these conditions, CXCR6⁺ ILCs that would be attracted to CD11b⁺ DCs would also receive signals promoting IL-22 production that could have a role in tissue regeneration and recovery from inflammation.

Several studies have investigated the cellular sources of IL-22 in mucosal tissues (reviewed in (Sonnenberg et al., 2011)). Early studies have demonstrated the importance of T cell receptor $\gamma\delta$ ⁺ cells that could be triggered by IL-1 β and IL-23 (Sutton et al., 2009). More recently, ILC that produce IL-22 have been implicated in protection against enteropathogens and in recovery following epithelial damage (Zenewicz et al., 2008; Zheng et al., 2008). Following *C. rodentium* infection, increased proportions of IL-22⁺ ILCs have been identified in the mLN, and in the crypts, villus LP, and epithelium of the small and large intestine (Cella et al., 2009; Lee et al., 2012; Satoh-Takayama et al., 2008; Sonnenberg et al., 2011).

We focused on the small intestine LP as this tissue harbors an abundant and diverse set of ILC subsets that produce IL-22. Our results demonstrate that CXCR6 plays an essential role in localizing IL-22-producing ROR γ t⁺ ILCs (especially NKp46⁺ ILC3) within the villus LP. This might be due to reduced ILC3 recruitment from the circulation or from poor maintenance of these cells once resident in the tissues. In contrast, the formation of ROR γ t⁺ ILC clusters remained intact. The consequences of this perturbed ILC topography included a marked reduction in overall IL-22 production and a corresponding decrease in the production of protective antimicrobial peptides by the intestinal epithelium. These observations suggest different mechanisms that control ILC3 distribution within the intestine and open the possibility for different roles for IL-22 production from ROR γ t⁺ ILCs present in the villus LP and the crypts.

Our results demonstrate the important role for localized IL-22 production in the villus LP in maintaining epithelial defense and identify CXCR6 expression as a key mechanism required for this function. IL-22 production in crypt structures might serve a similar function, and in addition, is proposed to influence ILF maturation (Ota et al., 2011) and subsequent antibody responses. The absence of CXCR6 resulted in a severe reduction in IL-22 expression. Still, in vitro culture of isolated LPLs demonstrated that IL-22 could be induced

(D) In vivo growth dynamics of bioluminescent *C. rodentium*. *Rag2*^{-/-}*Cxcr6*^{gfp/+} and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice were orally gavaged with 10⁹ bacteria and in vivo bacterial localization was determined by Xenogen imaging. Pseudo color images are shown (scale bar, photon/s/cm²/sr).

(E) Average radiance analysis. Data are compiled from three independent experiments with six mice of each genotype analyzed.

(F) Analysis of *Reg3g* and *Reg3b* expression by SI LPLs or SI IECs from *Rag2*^{-/-}*Cxcr6*^{gfp/+} and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice infected with *C. rodentium*. Three mice of each genotype were analyzed.

(G) IL-22 secretion by SI LPLs from *Rag2*^{-/-}*Cxcr6*^{gfp/+} and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice at steady-state or following infection with *C. rodentium*. Supernatants were collected after 24 hr culture without stimulation and IL-22 measured by ELISA.

(H) Effect of recombinant IL-22 on survival and loss of body weight in *C. rodentium*-infected *Rag2*^{-/-}*Cxcr6*^{gfp/+} (n = 6 mice) and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice (n = 6 mice each PBS or rIL-22 treated). 1 μ g of rIL-22 was injected intraperitoneally every 2 days postinfection. *p value < 0.05. **p value < 0.01. See also Figure S5.

in residual CD4⁺ and DN ILCs following stimulation with IL-23, and histological analysis revealed the presence of ROR γ ⁺ ILC3 clusters in the absence of CXCR6. It is possible that the triggers required for IL-22 production, such as IL-23, are not available in these clusters in vivo, thereby explaining the quasi absence of IL-22 expression after *C. rodentium* infection.

Previous reports have associated abnormal formation of lymphoid structures secondary to deficiencies in lymphotoxin- β receptor (LT β R) and deficiencies in IL-22 production by intestinal ILC subsets to defects in Notch signaling (Lee et al., 2012; Ota et al., 2011). The apparent normal development of lymphoid structures in CXCR6-deficient mice demonstrates that additional mechanisms control intestinal IL-22 production independent of organized lymphoid tissues. While a role for cryptopatches or ILFs in priming IL-22 production in intestinal ILC is possible (via LT β R-dependent IL-23 from DCs; Tumanov et al., 2011), our results demonstrate that CXCR6 expression is critical for IL-22⁺ ILC distribution throughout the villus LP. ILF are disorganized in *Ccr6*^{-/-} mice suggesting that CCR6 ligands attract and locally retain CCR6⁺ ILC3s (Sawa et al., 2010). Because CCR6⁻ NKp46⁺ cells do not accumulate in cryptopatches or ILFs (Sato-Takayama et al., 2011; Sato-Takayama et al., 2008; Sawa et al., 2010), our results highlight two independent chemokine pathways (CXCR6, CCR6) that orchestrate and segregate ILC3 subset localization within the intestine.

EXPERIMENTAL PROCEDURES

Mice

Cx3cr1^{gfp/+} (Jung et al., 2000), *Cxcr6*^{gfp/+} (Geissmann et al., 2005), *Rorc*^{gfp/+} (Sawa et al., 2010), and *Ncr1*^{gfp/+} mice (Gazit et al., 2006) were on the C57BL/6 background. All mice were housed under the specific pathogen-free condition at the Institut Pasteur, provided with food and water ad libitum and were analyzed at 8–18 weeks of age. Littermates were used for all experiments. Animal experiments followed the guidelines provided by the Animal Care Use Committee of the Institut Pasteur and were performed in accordance with French law (authorizations A7515-06, A7515-07, and 75-579).

Flow Cytometry Analysis

LPLs were extracted using collagenase digestions and density gradient centrifugation as described (Sato-Takayama et al., 2008). Cell surface and intracellular staining was performed as described (Sato-Takayama et al., 2011) with fluorochrome-coupled antibodies (Table S1). Epithelial cells were released using EDTA as described (Serafini et al., 2014). FACS Fortessa and AriaIII (BD Biosciences) were used for cell sorting and acquisition, respectively, and all flow cytometry data were analyzed with FlowJo (TreeStar). Mouse IL-22 ELISA was from R&D Systems.

Immunohistology

Immunohistological analysis was performed on formalin-fixed material and 8 μ m frozen sections were stained using fluorochrome-coupled antibodies as described (Sato-Takayama et al., 2008). For RegIII γ staining, endogenous peroxidase activity was quenched using PBS containing 0.3% H₂O₂. Sections were stained with rabbit anti-RegIII γ (Abgent), revealed peroxidase-coupled anti-rabbit immunoglobulin, and developed with DAB (Dako). Slides were counterstained with hematoxylin solution (Sigma-Aldrich).

Gene-Expression Analysis

RNA was isolated with the RNAeasy Micro Kit (QIAGEN) and amplified with MessageBooster (Tebu Bio). cDNA was generated with Superscript III Reverse Transcriptase (Invitrogen) and quantitative PCR performed with SYBR Green MasterMix (QIAGEN) with specific primers (EasyOligo; Sigma Aldrich; Table S2) or ChemokineSuperArray (QIAGEN). Samples were normalized to *Gapdh* expression.

Transwell Assay

Transwell chambers (5 μ m, Costar) were blocked with 1% BSA and coated with recombinant CXCL16 (R&D Systems). Total SI LPL (10⁶ cells) from *Rag2*^{-/-} *Cxcr6*^{gfp/+} or *Rag2*^{-/-} *Cxcr6*^{gfp/gfp} mice were plated in replicate in the upper chamber and cultured for 2 hr at 37°C. ILC3 subsets in the migrating cells were analyzed by FACS after cell surface staining as indicated above. Chemotaxis index indicates the migration ratio compared to wells without CXCL16.

C. rodentium Infection and Xenogen Imaging

Log phase cultures of BLI-*C. rodentium* (Wiles et al., 2006) were washed in PBS and mice were orally infected with 10⁹ cfu as described (Sato-Takayama et al., 2008). For in vivo tracking of *C. rodentium*, mice were anesthetized with ketamine (Imalgene 1000) and xylazine (Rompun). Prior to BLI, the abdominal region was shaved and mice were placed in a confinement box (TEM SEGA) and imaged using an IVIS 100 system (Caliper Life Sciences). Image acquisition and analysis were performed with the Living Image 3.2 software (Caliper Life Sciences) as described (Serafini et al., 2014).

Statistical Analysis

Experimental results are reported as mean \pm SEM. Statistical differences between groups were determined with the unpaired Student's t test. p values < 0.05 were considered significant. Significance for survival rates of Kaplan-Meier curves was calculated with the log rank test. All data were analyzed with Graphpad Prism software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2014.10.007>.

AUTHOR CONTRIBUTIONS

N.S.-T., N.S., and T.V. designed, performed, and analyzed experiments; A.R. contributed to setting up the Xenogen technology; J.-C. R. and G.F. provided critical reagents and advice; J.P.D. designed and directed the study; N.S.-T. and J.P.D. wrote the manuscript with help from all of the authors.

ACKNOWLEDGMENTS

We thank O. Mandelboim (Hebrew University, Jerusalem), F. Geissmann (Necker Hospital, Paris) and G. Eberl (Institut Pasteur) for *Ncr1*^{gfp/+} mice, *Cxcr6*^{gfp}/*Cx3cr1*^{gfp} mice, and *Rorc*^{gfp} mice, respectively. N.S.-T. was the recipient of fellowship from Japan Society for the Promotion of Science (JSPS), Japan. Supported by the Institut Pasteur, Inserm, and the ANR (Gut_ILC).

Received: June 13, 2013

Accepted: October 16, 2014

Published: November 6, 2014

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Supplemental Information

**The Chemokine Receptor CXCR6 Controls
the Functional Topography of Interleukin-22
Producing Intestinal Innate Lymphoid Cells**

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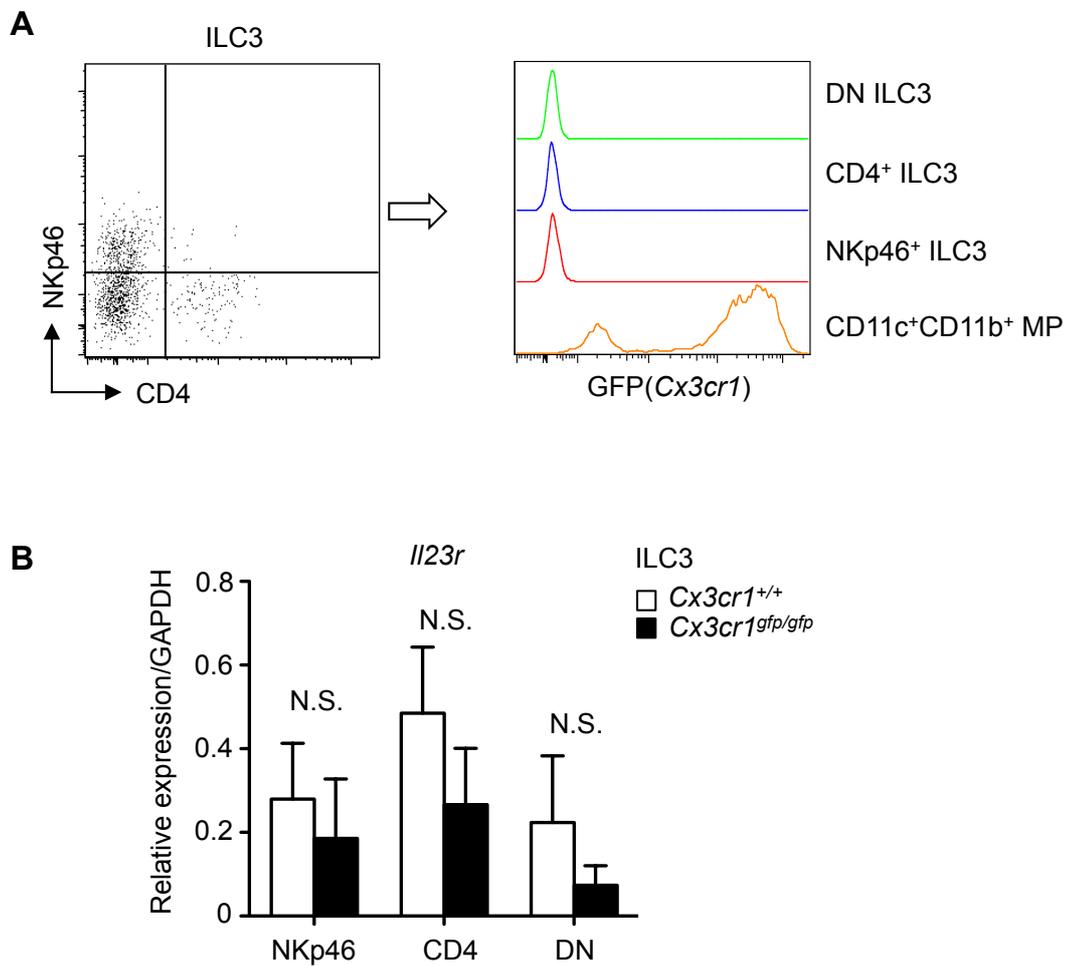


Figure S1, related to Figure 1 | ILC3 do not express *Cx3cr1*

(A) Analysis of *Cx3cr1* (GFP) expression in SI ILC3 subsets of *Cx3cr1*^{gfp/+} mice. Histograms show *Cx3cr1* expression in NKp46⁺, CD4⁺ and DN (NKp46⁻CD4⁻) ILC3 (CD3⁻CD127⁺RORγt⁺ cells). MHCII⁺CD11c⁺CD11b⁺ cells from SI LPL of *Cx3cr1*^{gfp/+} mice were used as positive control. (B) Analysis of *Ii23r* mRNA expression by NKp46, CD4 or DN ILC3 subsets in SI LPL from *Cx3cr1*^{+/+} and *Cx3cr1*^{gfp/+} mice. 3 mice of each genotype were analyzed.

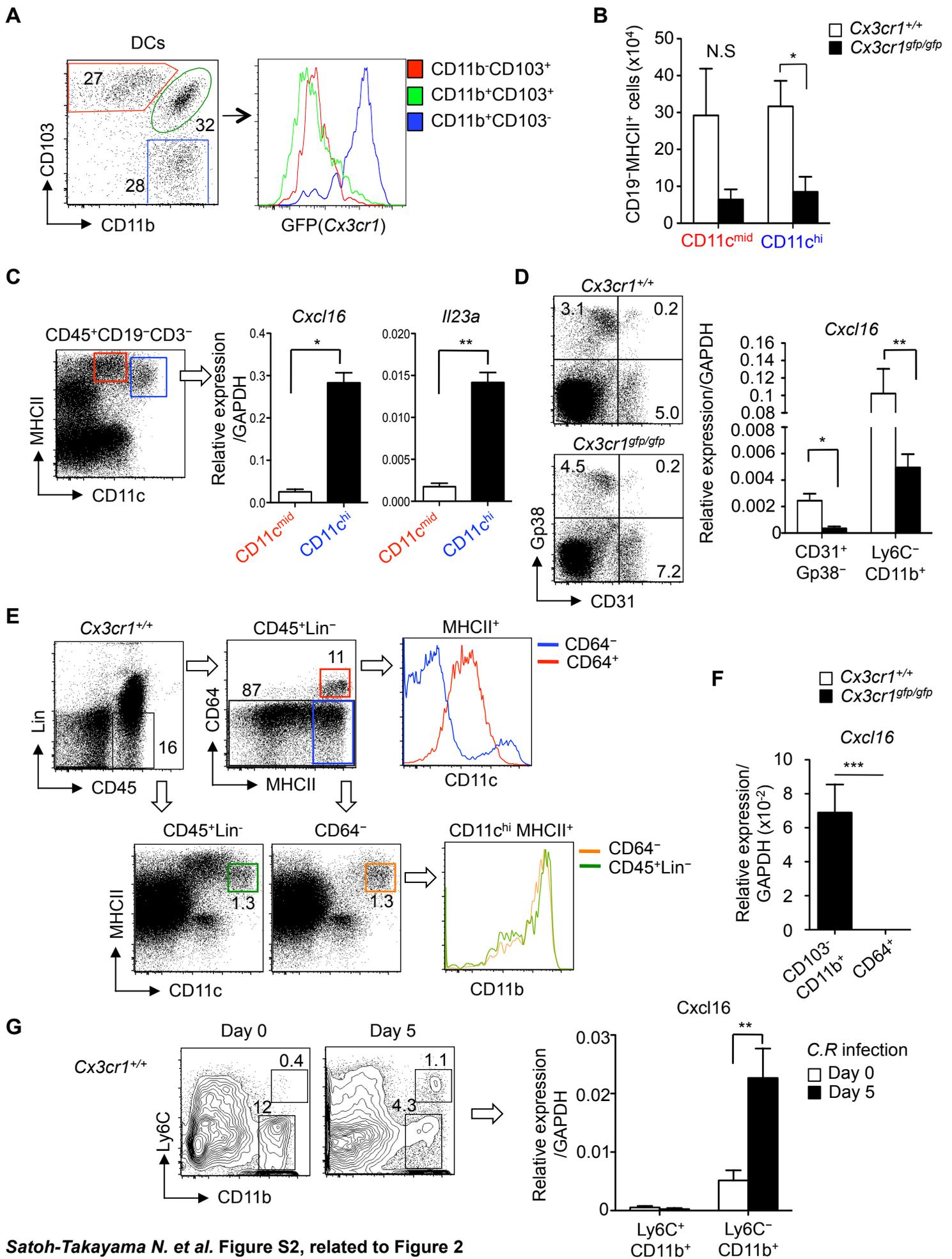


Figure S2, related to Figure 2 | Effects of CX3CR1 ablation on *Cxcl16* expression in intestinal myeloid and endothelial cells

(A) Analysis of *Cx3cr1* (GFP) expression in CD103 and/or CD11b expressing intestinal cDC subsets (MHC II⁺CD11c⁺ cells) in *Cx3cr1^{gfp/+}* mice. Data are representative of 3 independent experiments. **(B)** Absolute numbers of MHC II⁺CD11c^{mid} monocytes and MHC II⁺CD11c^{hi} cDC in SI LPL from *Cx3cr1^{+/+}* and *Cx3cr1^{gfp/gfp}* mice. 8 mice of each genotype were analyzed. **(C)** Analysis of *Cxcl16* and *Il23a* expression in sorted MHCII⁺CD11c^{mid} monocytes and MHCII⁺CD11c^{hi} cDC from wild-type mice. Data are representative of two independent experiments. **(D)** Analysis of *Cxcl16* expression by sorted intestinal endothelial cells (CD31⁺gp38⁻) in *Cx3cr1^{+/+}* and *Cx3cr1^{gfp/gfp}* mice. Ly6C⁻CD11b⁺ cells were used as positive control. 4 mice of each genotype were analyzed. **(E)** Analysis of monocyte and DC subsets by SI LPL in *Cx3cr1^{+/+}* mice. Comparison of CD11c expression in MHCII⁺ CD64⁺ cells (red line) with MHCII⁺ CD64⁻ cells (blue line) gated on CD45⁺Lin⁻ (CD3, CD19 and Ly6G) cells (upper part). Comparison of CD11b expression in CD11c^{hi} MHCII⁺ cells (green line) gated on CD45⁺Lin⁻ cells with CD11c^{hi}MHC II⁺ cells (orange line) gated on CD64 cells (bottom part). Representative results from 3 independent analyses. **(F)** Analysis of *Cxcl16* expression in CD103⁻CD11b⁺ expressing intestinal cDC subsets (MHCII⁺CD11c⁺ cells) and CD64⁺ expressing macrophage (CD45⁺Lin⁻MHCII⁺cells) in *Cx3cr1^{gfp/+}* mice. 4 mice of each genotype were analyzed. **(G)** Analysis of *Cxcl16* expression in inflammatory monocytes after *C. rodentium* infection (left panel). Ly6C⁺CD11b⁺ or Ly6C⁻CD11b⁺ cells were sorted at steady-state or 5 days after infection (right panel). 4 mice of each genotype were analyzed at each time point. *, *p* value < 0.05. ** *p* value < 0.01. ****p* value < 0.001.

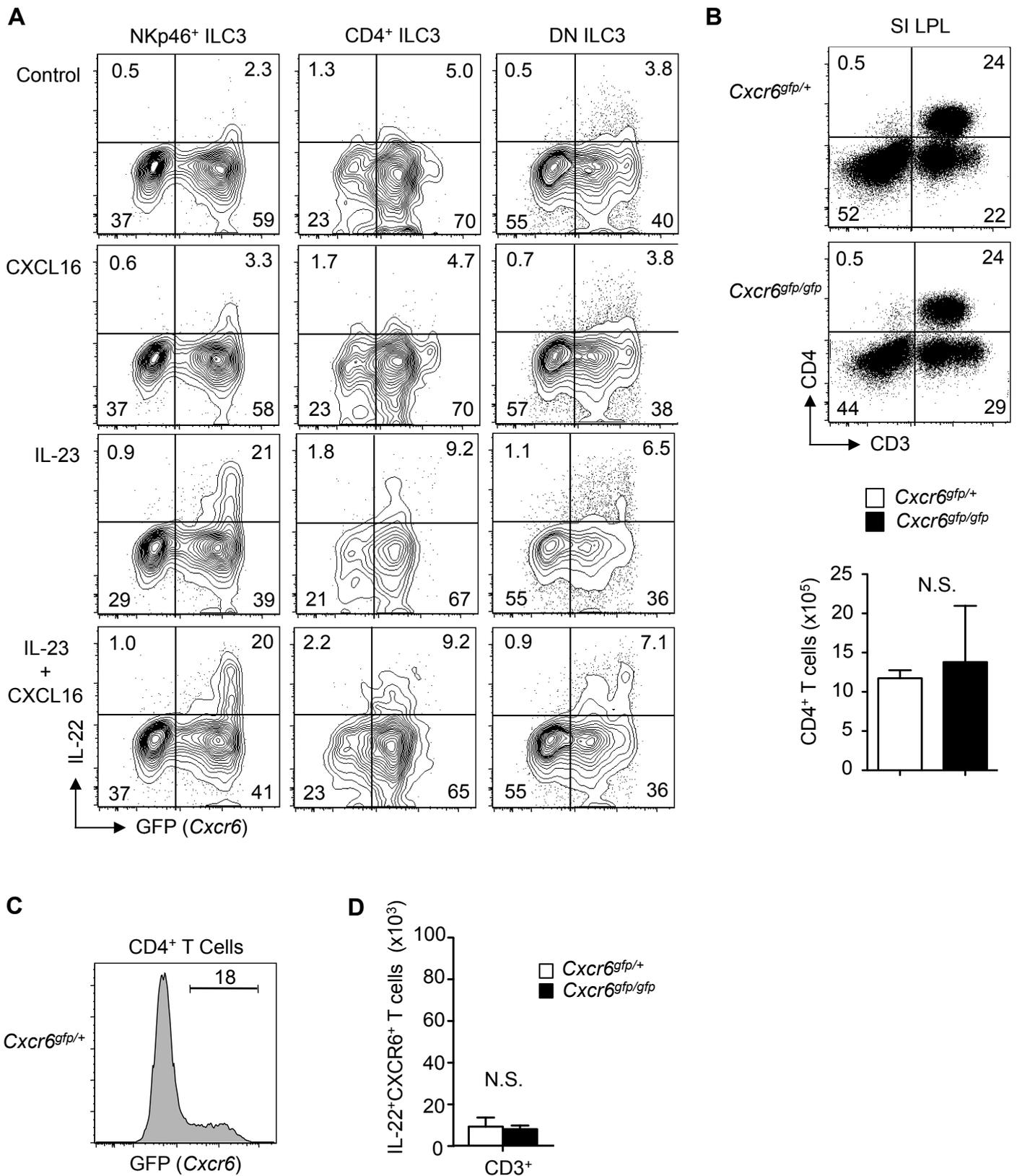


Figure S3, related to Figure 3 | Role of CXCL16 in regulating ILC3 and T cell homeostasis

(A) Analysis of IL-22 production versus *Cxcr6* (GFP) expression in ILC3 subsets from *Rag2*^{-/-}*Cxcr6*^{gfp/+} mice. SI LPL were stimulated with or without IL-23 and CXCL16 (100ng/ml) *in vitro* for 4 hrs. Data are representative two independent experiments. (B) Analysis of SI LPL T cells in *Cxcr6*^{gfp/+} and *Cxcr6*^{gfp/gfp} mice (left panel). Absolute numbers of CD3⁺CD4⁺ T cells were enumerated for 6 mice of each genotype (right panel). (C) Histogram shows *Cxcr6* (GFP) expression in CD3⁺CD4⁺ T cells in the SI LPL from *Cxcr6*^{gfp/+} mice. (D) Absolute numbers of intestinal IL-22⁺ T cells were enumerated for 4 mice of each genotype.

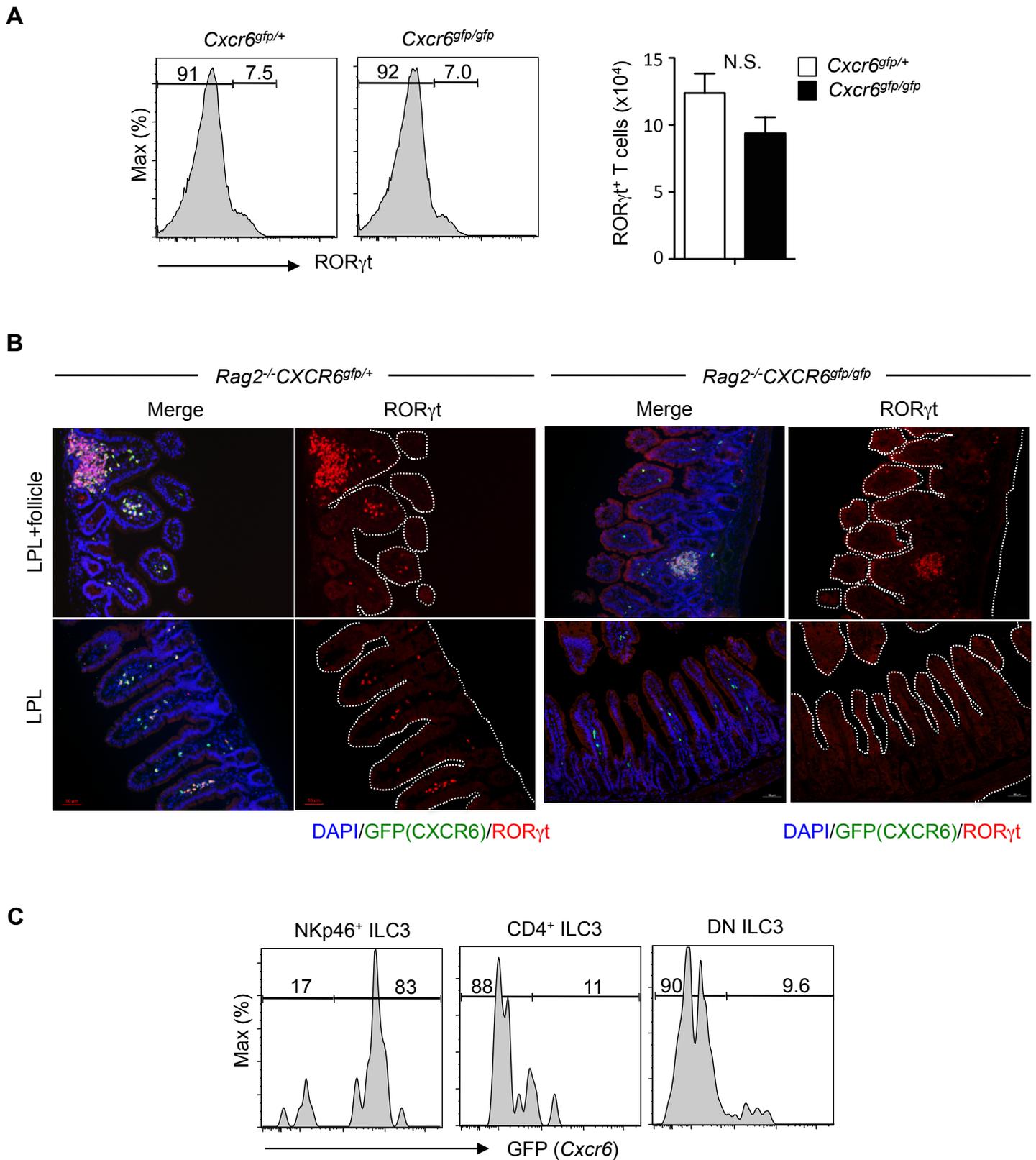


Figure S4, related to Figure 4 | T and ILC migration in *Cxcr6*-deficient mice

(A) Analysis of ROR γ t expressing T cells in the SI LPL from *Cxcr6*^{gfp/+} and *Cxcr6*^{gfp/gfp} mice (left panel). Absolute numbers of ROR γ t⁺ T cells were enumerated for 4 mice of each genotype (right panel). (B) Immunohistological analysis of CXCR6⁺ ILC3 in small intestine from *Rag2*^{-/-}*Cxcr6*^{gfp/+} and *Rag2*^{-/-}*Cxcr6*^{gfp/gfp} mice. Sections were stained with DAPI (blue), anti-GFP (CXCR6; green), anti-ROR γ t (red). Original magnification: x20. (C) Transwell migration assay using SI LPL by *Rag2*^{-/-}*Cxcr6*^{gfp/+} mice. For each ILC3 subsets, cells that migrated in response to CXCL16 were analyzed for GFP expression. Representative results from 4 independent experiments.

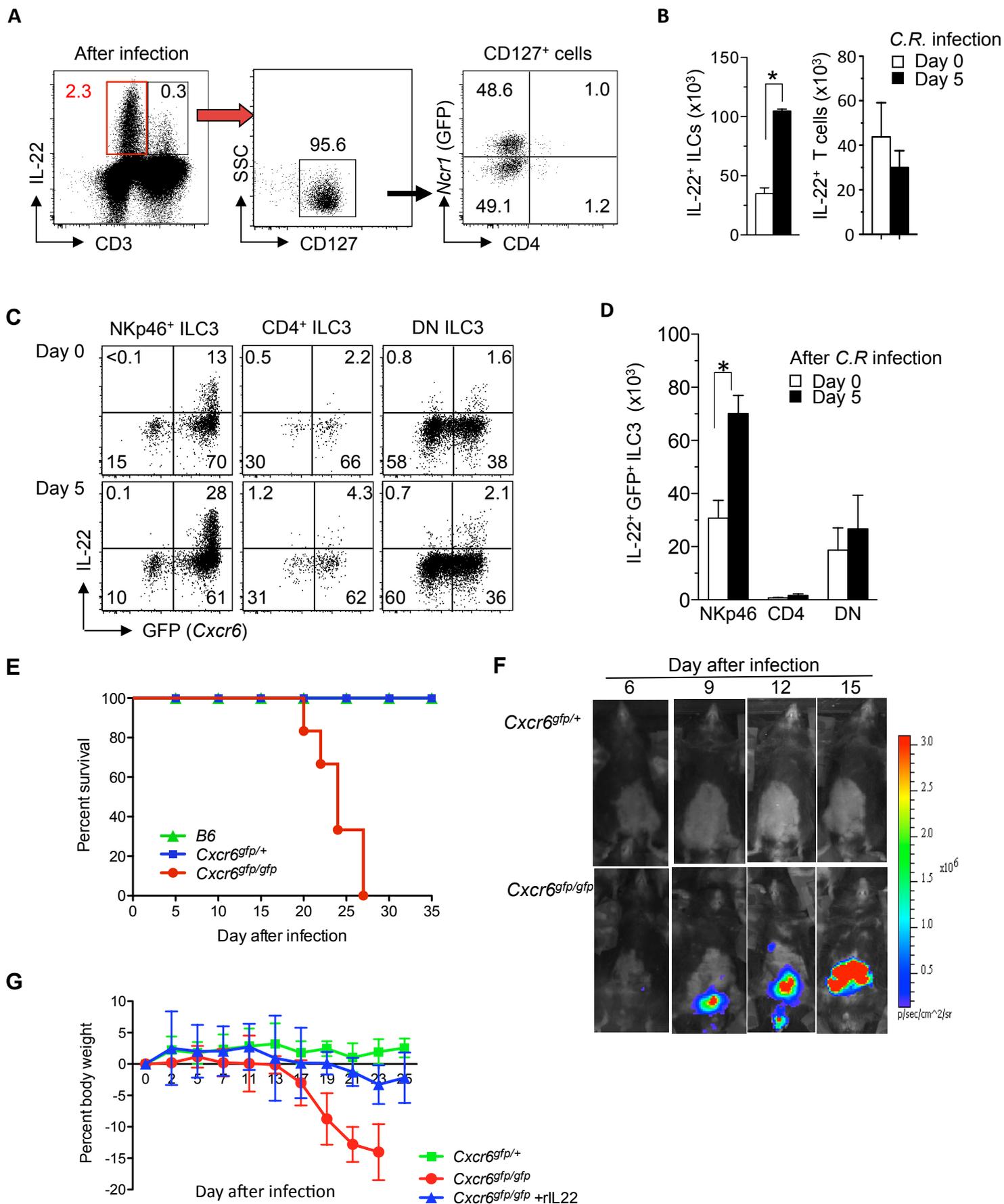


Figure S5, related to Figure 5 | CXCR6 required for *C. rodentium* immunity

(A) Analysis of IL-22-producing ILC after *C. rodentium* infection (day 5) in *Ncr1^{gfp}* mice. Boxes indicate percentage of IL-22⁺ CD3⁻ and CD3⁺ cells within the total population. **(B)** Absolute numbers of IL-22⁺ ILC (left panel) and IL-22⁺ T cells (right panel) in the SI LPL after *C. rodentium* infection. Four mice were analyzed at each time point. **(C)** Flow cytometric analysis of IL-22 producing ILC3 in SI LPL following *C. rodentium* infection. *Cxcr6^{gfp/+}* mice were orally gavaged with 1×10^9 bacteria. **(D)** Absolute numbers of IL-22 producing ILC3 subsets in *Cxcr6^{gfp/+}* mice at steady-state and after *C. rodentium* infection (5 days post-infection). Seven mice of each genotype were analyzed. **(E)** Survival after *C. rodentium* infection in C57Bl/6, *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice. Mice were orally gavaged with 1×10^9 bacteria. Data are compiled from 4 independent experiments (total mice n=6 for C57BL/6, n=9 for *Cxcr6^{gfp/+}*, n=9 for *Cxcr6^{gfp/gfp}*). **(F)** *In vivo* growth dynamics of bioluminescent *C. rodentium*. *Cxcr6^{gfp/+}* and *Cxcr6^{gfp/gfp}* mice were orally gavaged with 10^9 bacteria and *in vivo* bacterial localization was determined by Xenogen imaging. **(G)** Effect of recombinant IL-22 on loss of body weight in *Cxcr6^{gfp/+}* (n = 6 mice) and *Cxcr6^{gfp/gfp}* mice (n = 6 mice each PBS or rIL-22 treated). 1mg of rIL-22 was injected *i.p.* every 2 days post-infection. *, *p* value < 0.05.

Table S1, related to Experimental Procedures | Antibodies for FACS analysis and IHC

Antibodies	Clone	Company
CD3	17A2	eBioscience
CD3	500A2	eBioscience
CD19	1D3	BD Bioscience
CD45.2	104	eBioscience
CD11b	M1/70	BD Bioscience
CD11c	N418	eBioscience
CD103	M290	BD Bioscience
IA/IE	M5/114.15.2	eBioscience
CD127	A7R34	eBioscience
NKp46	29A1.4	eBioscience
IL-22	1H8PWSR	eBioscience
ROR γ t	AFKJS-9	eBioscience
CD90.2	53-2.1	eBioscience
CD4	RM4-5	BD Bioscience
NK1.1	PK136	Biologend
RegIII γ	pAb #AP5606c	Abgent
gp38	eBio8.1.1	eBioscience
CD31	390	eBioscience
CD64	X54-5/7.1	BD Bioscience
Ly6G	1A8	eBioscience
Ly6C	HK1.4	eBioscience

Table S2, related to Experimental Procedures | Primers sequences used for qPCR

Gene name	Forward primers (5'-3')	Reverse primers (5'-3')
<i>Il22</i>	TTGAGGTGTCCAACCTCCAGCA	AGCCGGACGTCTGTGTTGTTA
<i>Cxcl16</i>	CCTTGTCTCTGGCGTTCTTCC	TCCAAAGTACCCTGCGGTATC
<i>Reg3g</i>	CCGTGCCTATGGCTCCTATTG	GCACAGACACAAGATGTCCTG
<i>Reg3b</i>	ACTCCCTGAAGAATATACCCTCC	CGCTATTGAGCACAGATACGAG
<i>Gapdh</i>	AGGTCCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<i>Il23a</i>	ATGCTGGATTGCAGAGCAGTA	ACGGGGCACATTATTTTTAGTCT
<i>Rorc</i>	ACCTCCACTGCCAGCTGTGTGCTGTC	TCATTTCTGCACTTCTGCATGTAGACT
<i>Lcn2</i>	GCAGGTGGTACGTTGTGGG	CTCTTGTAGCTCATAGATGGTGC
<i>Il23r</i>	TTCATATGGGCATGAATGTTTCT	CCAAATCCGAGCTGTTGTTCTAT

Article n°2

The transcription $ROR\gamma t$ instructs cytokine expression, tissue positioning and cytokine responsiveness of group 3 Innate Lymphoid Cells (ILC3). Remarkably, $NKp46^+$ ILC3 concomitantly express and depend on T-bet, GATA3 and $ROR\gamma t$. While T-bet expression drives ILC3 production of $IFN-\gamma$, $ROR\gamma t$ controls IL-22 secretion in these cells.

$NKp46^+$ ILC3 are reported to modulate $ROR\gamma t$ expression⁷⁰, thereby converting into cells that are indistinguishable from intestinal ILC1. In this case, this modulation reflects the plasticity of $NKp46^+$ ILC3 and their ability to adapt to their environment.

$NKp46$ expression is a common feature between ILC3 and ILC1, and most likely reflects the conserved expression of T-bet. Indeed, haploinsufficiency in $ROR\gamma t$ is associated with an increase in both $NKp46$ and T-bet expression by $NKp46^+$ ILC3²⁴⁸, bringing compelling evidence that regulation of these different transcription factors is intertwined.

Whether $NKp46^+$ ILC3 also modulate $NKp46$ expression is not known. To answer this question, we assessed stability of $NKp46$ expression using a fate-mapping approach in mice. We characterize $NKp46^-$ ILC3 with a previous history of $NKp46$ expression. This work demonstrates a novel phenotypic and functional plasticity within the $NKp46^+$ ILC3 subset.

Diffusion Version

**Article N°2 : Phenotypic and Functional Plasticity of Murine
Intestinal NKp46⁺ Group 3 Innate Lymphoid Cells**

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James P. Di Santo and Christian A. J. Vosshenrich

J Immunol June 1, 2016, 196 (11) 4731-4738;

Download at <https://doi.org/10.4049/jimmunol.1502673>

Article n°3

Notch signaling instructs cell-fate decisions from early embryonic stages onwards (for review see²⁵²). In the hematopoietic compartment, development of intestinal dendritic cells, generation of marginal zone B cells and most importantly the extinction of alternative fate in T cell precursors largely depend on this signaling pathway.

Innate lymphoid cells (ILC) are seen as innate counterpart of T cells, as they display similar effector function without somatically rearranging receptor. The close resemblance between T cells and ILC might account for their shared dependency toward Notch-signalling.

In vitro culture of common lymphoid progenitors revealed that Notch-dependent blockage of B cell promoted the generation of $\alpha 4\beta 7^+$ cells¹²⁷, which are known to contain ILC committed precursors. *Rbpjk*^(Δ VAV) mice, in which all hematopoietic cells are unable to transduce notch-signalling, exhibit a marked reduction in NKp46⁺ ILC3 numbers²⁴⁶. Because Notch plays diverse role in the hematopoietic compartment, several defect could account for the phenotype observed in *Rbpjk*^(Δ VAV) mice.

The last paper presented in this thesis aim to dissect the role of Notch in NKp46⁺ ILC3 homeostasis. We confirmed the cell-intrinsic role for Notch signaling in NKp46⁺ ILC3 in different mice models, and extended our observations by assessing gene expression impacted by Notch-deficiency.

Article N°3 : Notch signaling in group 3 innate lymphoid cells modulates their plasticity

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Sci. Signal. 03 May 2016:

Vol. 9, Issue 426, pp. ra45

Download at : DOI: <http://stke.sciencemag.org/content/9/426/ra45/tab-pdf>

“ONTOGENY OF Eomes independent NKp46⁺ ILC: how, why, what, because maybe”

I contributed to the first paper presented in this thesis, which presented the crosstalk between CX3CR1⁺ dependent myeloid cells and NKp46⁺ ILC3. Briefly, CX3CR1⁺ CD11b⁺ are the main producer of CXCL16, which ensures the positioning of NKp46⁺ ILC3 in a CXCR6 dependent manner into the villi of the lamina propria, and produce IL-23, the IL-12 member that drives IL-22 production by ILC3. NKp46⁺ ILC3-derived IL-22 is then ensured by CX3CR1⁺ CD11b⁻ secretion of TL1A and IL-1 β in response to microbiota¹⁴². Most importantly, CX3CR1⁺ CD11b⁻ also arise post natively, similarly to NKp46⁺ ILC3, indicating that these immune populations are intricately linked. Although I made use of TGF β and Notch in my article, discussing their role would require introducing other concepts, most of which have already been discussed in third article. CD103⁺ CD11b⁻ Dendritic cells are reported to produce Retinoic Acid²⁵³, which induces Rorc expression, but also TGF β and Notch ligands. In addition, CD103⁺ CD11b⁻ Dendritic cells are another source of IL-23, which induces IL-22 production by NKp46⁺ ILC3. Overall, the interaction between NKp46⁺ ILC3 and the different myeloid subsets directs their localization, and provides signals important for their regulation and activation.

In this final section, I will mainly focus on the second paper that represents the larger part of my PhD work. I will therefore present different factors that control the Ncr1^{FM} ILC3 phenotype and functions. Ncr1^{FM} ILC3 have an history of Ncr1 expression, express higher amount of IL-22, but not IFN- γ , indicating that they lost some of the ILC1^a associated features. These factors will be then replaced in the network determining ILC1^a vs ILC3 fate decisions.

Ncr1 promoter activation in ILC3 is T-bet independent

Another report by Viant and colleagues²⁵⁴ identified Ncr1^{FM} ILC3, which comforts my observations. I pointed out that T-bet level in Ncr1^{FM} was lower than that of NKp46⁺ ILC3 (Fig2H), but higher than CD4⁺ ILC3. As Ncr1^{FM} are an heterogeneous population that comprises CD49a⁺ T-bet⁺ and CCR6⁺ T-bet⁻, intermediate level of T-bet in Ncr1^{FM} could be a combination of the T-bet⁺ and T-bet⁻ fraction. Viant et al. reported that half of Ncr1^{FM} expressed T-bet. In their study, they backcrossed NFM mice to a *Tbx21* deficient background, in which NKp46⁺ ILC3 are virtually absent. Still, a fraction of Ncr1^{FM} ILC3 persisted, indicating that activation of the *Ncr1* promoter is independent of T-bet expression in ILC3. Knowing that *Tbx21* deficient mice lack all T-bet⁺, which include NKp46⁺ and NKp46⁻ ILC3, but neither CCR6⁺ ILC3 nor CCR6⁻ T-bet⁻ ILC3, this indicates that the residual Ncr1^{FM} ILC3 present in *Tbx21* deficient mice are probably CCR6⁺ ILC3 and CCR6⁻ T-bet⁻. I also reported the presence of Ncr1^{FM} in one week-old mice (Fig3A),

in which NKp46⁺ ILC3 have not developed yet, and contain mostly T-bet⁻ CCR6⁺ and CCR6⁻ ILC3. The heterogeneity observed in Ncr1^{FM} ILC3 implies that another factor common to ILC3 drive *Ncr1* promoter activation.

Runx3 binds the *Rorc* promoter in ILC3

Runx3 is a member of the Runx family of transcription factor, which includes Runx1, Runx2 and Runx3 (for review in T^H cells commitment see Collins et al. 2009²⁵⁵, or in cancer development see Lai & Mager 2012²⁵⁶). Both Runx1 and Runx3 require CBF- β to exert their transcriptional activity. Runx3 is expressed in mature ILC1^a and ILC3, but neither in ILC2 nor in ILC1^b. *Runx3* ^{Δ VAV} mice are devoid of intestinal ILC1^a and ILC3, while the ILC2 compartment remain unaffected²⁴⁸. When transfected with a vector expressing luciferase driven by the complete promoter of the mouse *Rorc*, NK-92 cells, a human ILC1^b-cell line characterized by high expression of human Runx3, efficiently expressed luciferase, which expression was abrogated upon deletion of a Runx3 putative binding site in the mouse *Rorc* promoter²⁴⁸. In addition, Runx3 binds the *Rorc* promoter in isolated ILC3, which comforts the putative role for Runx3 in controlling ROR γ t expression in ILC3. In NK-92 cells, human Runx3 also binds the endogenous *Ncr1* promoter, while mouse NK cell line transfected with a vector expressing dn-Runx, which acts as a Runx negative competitor for DNA-binding, possess lower transcripts for *Ncr1*²⁵⁶. As Runx3 is expressed by all ILC3, and is able to bind the *Rorc* and *Ncr1* promoter, this transcription factor could act as an activator of the *Ncr1* promoter in ILC3.

Lin⁻ GATA-3^{int} T-bet⁺ Cells are present in *Cbfb* Δ NKp46 mice

Intestinal ILC1^a and NKp46⁺ ILC3 are both GATA3^{int} and T-bet⁺(84, 248), and are virtually absent in *Runx3* ^{Δ NKp46} and *Cbfb* ^{Δ NKp46} mice(248), indicating that mature intestinal ILC1^a and ILC3 intrinsically require the Runx3/CBF- β complex for their generation. Interestingly, an intestinal population that is Lin⁻ GATA3^{int} CD127⁺ is overrepresented in *Runx3* ^{Δ VAV} mice, while *Cbfb* ^{Δ NKp46} mice harbor a population of Lin⁻ GATA3^{int} T-bet⁺ NKp46⁻ ROR γ t⁻. As *Cbfb* ^{Δ NKp46} mice still express Runx3, which presumably binds both the *Rorc* and *Ncr1* promoter, NKp46⁺ ILC3 maturation appear to be initiated by Runx3 and stabilized by CBF- β .

A subset of intestinal ILC1a express *Rorc* and AhR

In ROR γ t^{GFP/GFP} mice, some ILC^a express GFP⁽¹²³⁾, indicating that ROR γ t is dispensable for its own expression in ILC1^a. Some ILC1^a are fate-mapped for ROR γ t⁷⁰, which, upon transfer in RAG^{KO/KO} *Il2rg*^{KO/KO} upregulates ROR γ t, indicating that *Rorc* expression is modulated in ILC1^a. In addition, transcriptomic analysis revealed that ILC1^a express both *AhR* and *Il21r*³⁷. In T^H cells, IL-21, in combination with low amount of TGF β , is sufficient to induce IL-22 production^{257,258}, as well as *Il23r* and *Il1r* expression, but neither T-bet nor IL-17 expression *in vitro*. In addition, culture of ILC1^b with IL-21 led to a marked downregulation of NK1.1²⁵⁹. *In vitro* culture human NK-cells with L-kynurenine, a tryptophan metabolite that binds AhR²⁶⁰, prevented IL-2/15-induced NKp46 upregulation. Both NKp46⁺ and NK1.1⁺ expression are lower in ILC3 when compared to ILC1. Moreover, NKp46⁺ ILC3 only produce IL-22, not IL-17, thereby resembling IL-21-induced T^H22 producing cells. One possibility is that ILC1^a exhibits the potential to upregulate ROR γ t, reduce their surface expression of NKp46 and NK1.1 in response to AhR ligands and IL-21, respectively. In these cells, IL-21 could upregulate *Il23r* and *Il1r*, which would stabilize ROR γ t expression and permit IL-22 production in response to IL-1 β . Finally, AhR was reported to directly bind the *Ckit* promoter⁽¹⁹⁸⁾. Upon stimulation with AhR ligands, isolated ILC3 increased their CD117 surface expression⁽¹⁹⁸⁾, indicating that CD117 expression can be induced in ILC1^a. Stabilized ROR γ t expression would eventually be required to generate mature NKp46⁺ ILC3 cells. This pathway would explain the shared dependency of ILC1^a and NKp46⁺ on T-bet.

Interplay between GATA-3, T-bet and ROR γ t

CCR6⁻ ILC3 from *Tbx21*^{KO/KO} T-betZSGREEN mice are ZSGREEN⁺, indicating that T-bet is dispensable for its own expression in CCR6⁻ ILC3²⁴⁸. NKp46 expression in ILC3 from *Rorc*^{KO/+ Δ T-BET} mice is higher than that in *Rorc*^{+/+ Δ T-BET} mice, demonstrating that haplodeficiency in *Rorc*, and presumably lower intracellular amounts of ROR γ t, impacts NKp46 expression. In *Tbx21* deficient mice, the amount of ROR γ t in ILC3 was similar to that of WT mice, while *Gata3* ^{Δ RORC} ILC3 exhibit higher level of intracellular ROR γ t, implying that GATA3, but not T-bet influences ROR γ t expression. As GATA3 binds the *Rorc* promoter in ILC3, it appears likely that GATA3 directly represses *Rorc* expression. CCR6⁻ ILC3 from *Tbx21* deficient mice express lower level of CD127, indicating that T-bet intracellular level impacts their expression. CD127 expression was also reduced following inducible deletion of GATA3 in mature ILC3²⁴⁸, which, added to the fact that GATA3 directly bind the *Il7ra* promoter, indicates that GATA3 controls CD127 expression.

Intracellular amount of Runx3 directs TH1 versus TH2 decision

In TH2 polarized cells, overexpression of Runx3 lead to an increase in *Tbx21* transcripts and IFN- γ production while reducing IL-4 production without affecting GATA3 expression²⁶¹. In addition, GATA3 and Runx3 physically interact, forming a GATA3/Runx3 complex that binds DNA. Runx3 also reduced GATA3 induced *Il5* expression, an effect that required GATA3 binding to the *Il5* promoter. In this case, the GATA3/Runx3 complex bound and directly repressed GATA3 regulated gene, and high expression of Runx3 was associated with an inhibitory effect on TH2 function and activation of TH1 gene set. In TH1 polarized cells, Runx3 was directly reported to bind the *Ifng* promoter²⁶². In HEK293T cells, a human T cell line, transfected with a vector in which the C-Terminal portion of GATA3 is deleted, the GATA3/Runx3 could suppress IFN- γ production, but failed to induce *Il4* expression, indicating that the C-Terminal portion mainly control the inductive function of GATA3. Deletion of the N-Terminal portion of GATA3 abrogated the binding to Runx3, which was required to repress IFN- γ production. The ability of the Runx-3 to directly bind and regulate the activating and repressing function of GATA3-mediated transcription points that it acts as rheostat to tune the transcription of T-bet target genes. If this is also true in ILC3, the Runx3/GATA3 complex, in addition to directly impacts *Ncr1* and *Rorc* expression, might control T-bet induced genes, i.e. *Ifng*, and therefore regulates the balance between NKp46⁺ ILC3 and ILC1^a. Increased amount of Runx3 would inhibit GATA3 induced genes such as *Il7ra* and *Rorc*, and promotes a T-bet driven genes, including *Ifng*. As such low level of surface NKp46 expression in ILC3, which is directed by Runx3, marks an intermediate “phenotype” between ILC1^a and ILC3.

Intracellular amount of Runx1 directs TH1 versus TH17 decision

Runx1, which also requires CBF- β to exert its transcriptional activity, operates in a similar fashion as Runx3. While Runx3 controls the balance between TH1 and TH2, Runx1 rather controls TH1 versus TH17 decisions. Runx1 is both able to interact with T-bet and to bind the *Ifng* and the *Rorc* promoters⁽⁹¹⁾. In HEK293T that were transfected with a vector expressing Luciferase under the control of the *Rorc*, increased amounts of Runx1 correlated with increased luciferase activity, while co-transfection with T-bet abrogated Runx-1 induced *Rorc* activation. These results imply that high amount of Runx1 induces *Rorc* expression and that the Runx1/T-bet complex prevents *Rorc* expression. Remarkably, TH1 cells cultured with TGF β express Runx1, and upregulate genes associated with a TH17 function, including *Rorc*²⁶³. Invalidation of Runx1 abrogates TGF β induced *Rorc* upregulation, indicating that TGF β participates in the upregulation

of *Rorc*. It is also possible, in the Runx1/T-bet, similarly to the Runx3/GATA3, the amount of Runx1 dictates the activation and repression of T-bet target genes.

Concluding remarks



Fig 25 : Runx3 and Runx1 antagonize ILC1^a and ILC3 associated genes

In *Cbfb*^{ΔNKp46} mice, an immature population of Lin⁻ GATA3^{int} T-bet⁺ NKp46^{lo} RORγt^{lo} is over-represented, and will be referred to as GTNR cells. I will use this population as a basis to describe the network of factors that drive ILC1^a versus T-bet⁺ ILC3. GTNR cells represent an intermediate stage that develops in the absence of Runx protein signaling, and contain the potential to generate both ILC1^a and T-bet⁺ ILC3. Because deletion of CBF-β occurs after NKp46 expression, which supposedly is a late in event T-bet⁺ ILC3, GTNR do not reflect the “conventional ontogeny”, but rather marks the “closeness” of ILC1^a and T-bet⁺ ILC3.

Runx proteins are not required for T-bet expression in GNTN cells, but required for ROR γ t expression. Runx3 is present in ILC precursors, and therefore precedes T-bet expression and ROR γ t expression. Nonetheless, there appears to be a branching between T-bet and ROR γ t expression. In *TGFBRA*^{ΔNKp46} mice, constitutive TGF β signaling leads to decreased amounts of ILC1^a and NKp46⁺ ILC3²⁵⁴, implying that TGF β antagonizes T-bet-associated fate, which could be due to increased amounts of Runx1. Although mature ILC3 do not express Runx1, CD4⁺ ILC3 are absent from the fetal intestine in the context of Runx1 deficiency, indicating that Runx1 expression is required at the early stage of ILC3 ontogeny. In T^H cells, IL-27, a member of the IL-12 family, induces T-bet expression as well as IL-21 production, which would participate in AhR induction in an autocrine fashion. As GNTN retain potential for generating ILC1^a and T-bet⁺ ILC3, all of which express AhR and T-bet, IL-27 appears as an interesting candidate for triggering *Tbx21* expression, and would participate in an eventual conversion from ILC1^a to NKp46⁺ ILC3. In addition, the relative amount of IL-12 and IL-23 in the environment, surely controls the stabilization of ILC1^a, T-bet⁺ ILC3 and CCR6⁺ ILC3 phenotypes by regulating *Tbx21* and *Rorc* intracellular amounts.

Overall, Lin⁻ GATA3^{int} CD127⁺ cells that reside in the intestine are the progenitor for ILC3 and ILC1^a. At early stages of development, intestinal TGF β triggers Runx1, which prevents T-bet from exerting its transcriptional activity, and, in combination with RA and Runx3, permits *Rorc* expression, thereby leading to the generation of CD4⁺ ILC3 from Lin⁻ GATA3^{int} CD127⁺. However, CCR6⁻ ILC3 are reported to arise around 2 weeks of age, while Ncr1^{FM} are detected in 1-week-old mice. Assessing whether Ncr1^{FM} express CCR6 in 1-week-old mice would instruct us as to whether the CCR6⁺ ILC3 fraction contains cells that retain the potential to convert into T-bet⁺ ILC3, but are phenotypically undistinguishable from CCR6⁺ because of T-bet-antagonizing effects of TGF β signaling. Remarkably, fate-mapping studies revealed that a fraction of CCR6⁺ express PLZF, which also marks NKp46⁺ ILC3, further suggesting heterogeneity in the CCR6⁺ ILC3 compartment.

In the model I propose, which does not reflect the ontogeny, Runx3/GATA3 complex controls the ILC1^a associated markers by repressing or activating T-bet target genes. GATA3 expression appears stable in ILC1^a and ILC3, and functions as a transcriptional “adaptor”. As *Ncr1* is a direct target of Runx3, NKp46 surface expression, and Ncr1 fate-mapping, reflects the transcriptional activity of the Runx3/GATA3 complex. In NKp46⁺ cells (either ILC1^a or ILC3), the Runx3/GATA3 complex might allow for the expression of T-bet-expressing genes, while in NKp46^{FM} ILC3 the Runx3/GATA3 complex allows

for *Gata3* driven expression genes, i.e. CD127. TGF β -induction of Runx1 represses T-bet associated genes, which lead cells to the acquisition of a CCR6⁺ ILC3 phenotype. Therefore, the balance between the Runx3/GATA3 and Runx1/T-bet complexes tunes ILC1^a versus Ncr1^{FM} CCR6⁺ ILC3 fate and functions. This balance is regulated by the responsiveness to IL-12 members. Establishment of a “stable” phenotype is probably the result of sustained signaling, implying epigenetic modification and chromatin remodeling of the locus that encodes transcription factors (*Rorc*, *Tbx21*, *AhR*) and cytokines (*Il22*, *Ifng*).

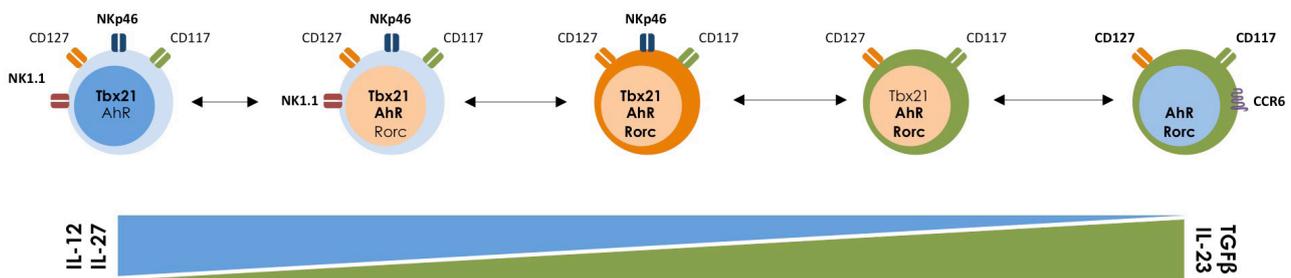


Fig 26 : Cytokine Responsiveness dictate ILC1^a versus ILC3 fate decisions

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Les cellules lymphoïdes innées de groupe 3 (ILC3) contribuent activement à l'homéostasie intestinale par leur production d'Interleukin-22 (IL-22). Ces ILC3 regroupent 2 sous populations majeures, les LTi (« Lymphoid Tissue inducer »), caractérisées par l'expression du récepteur au chimiokine CCR6, et les ILC3 exprimant le facteur de transcription (FT) T-bet, qui comprennent une population positive pour le marqueur de surface NKp46, récepteur originalement utilisé pour identifier les ILC de groupe 1 (ILC1). Les ILC1 jouent un rôle prépondérant dans la réponse aux pathogènes intracellulaires et anti-tumorale. Jusqu'à présent, trois populations majeures composent les ILC1: les lymphocytes cytotoxiques Natural Killer (NK ou ILC1b), qui dépendent largement du FT Eomes et expriment l'intégrine CD49b; les ILC1 hépatiques et intestinaux, qui dépendent du FT T-bet et expriment CD49a (ILC1a); et une population CD49a+ et DX5+ indépendante du FT Nfil3 localisée dans les glandes salivaires ou l'utérus (ILC1ab).

Mes travaux visent à comprendre la biologie des ILC exprimant NKp46, ainsi que les facteurs impliqués dans leur développement, leur maturation et leur fonction.

La majeure partie de ma thèse se concentre sur les NKp46+ ILC3. Premièrement, nous démontrons un rôle majeur pour le récepteur aux chimiokines CXCR6 dans la localisation des NKp46+ ILC3 dans les villi de la lamina propria intestinale (Satoh-Takayama et al. 2014). Deuxièmement, j'ai mis en évidence que NKp46+ ILC3 pouvait perdre l'expression de NKp46 (Verrier et al. 2016). Déclenchée par le TGF β , cette perte d'expression est associée à une plus forte capacité à produire de l'IL-22, mais aussi à l'acquisition de marqueurs identifiant les LTi (CCR6, MHC-II), démontrant ainsi la plasticité des NKp46+ ILC3. Enfin, en collaboration avec le groupe de Rachel Golub, nous avons confirmé le rôle présumé de la molécule Notch dans cette plasticité (Chea et al. 2016).

Dans ce manuscrit, je discuterai du développement et de l'hétérogénéité des ILC3, ILC1a, ILC1b et ILC1ab.

L'ensemble de mes résultats soutient une vision dynamique de la biologie des ILC reflétant l'adaptation de ces cellules effectrices face à leur environnement. En caractérisant les différents acteurs impliqués dans ce processus dynamique, mes travaux pourront servir au développement de thérapies visant à contrôler l'équilibre entre ces différentes populations dans diverses pathologies comme le cancer, les infections virales, ou encore les maladies intestinales.

Group 3 Innate Lymphoid cells (ILC3) actively maintain mucosal homeostasis through the production of Interleukin-22 (IL-22). ILC3 encompass 2 major populations, LTi (« Lymphoid Tissue inducer »), characterized by the expression of the chemokine receptor CCR6, and ILC3 that express the transcription factor T-bet, which include a population expressing the surface marker NKp46, a receptor originally used to identify group 1 ILC (ILC1). ILC1 plays a major role in the defense against intracellular pathogens and anti-tumoral responses. Three major ILC1 populations have been identified: the cytotoxic lymphocytes « Natural Killer » (NK or ILC1b), which largely rely for on the transcription factor Eomes their generation and express the integrin CD49b; hepatic and intestinal ILC1 that depends on the T-bet transcription factor and express CD49a (ILC1a); and a population that expresses CD49a and CD49b (ILC1ab) and populates the salivary gland and the uterus, which is independent of the transcription factor Nfil3.

My work aimed to understand the biology of NKp46 expressing ILC, as well as factor involved in their development, maturation and function.

The major part of my work focuses on NKp46+ ILC3. First, we demonstrate a major role for the chemokine receptor CXCR6 in their localisation in the lamina propria vili (Satoh-Takayama et al. 2014). Second, I showed that NKp46+ ILC3 could lose NKp46 expression (Verrier et al. 2016). Induced by TGF β , this loss of expression was associated with higher IL-22 production and by the acquisition of markers identifying LTi (CCR6, MHC-II), demonstrating NKp46+ ILC3 plasticity. Finally, in collaboration with Rachel Golub's group, we confirmed a putative role for Notch-signaling in this plasticity (Chea et al. 2016).

In this manuscript, I will discuss the development and the heterogeneity of ILC3, ILC1a, ILC1b and ILC1ab.

All the results I generated support a dynamic vision of ILC biology, which reflects how they adapt in response to environmental cues. By characterizing the different actors involved in this dynamic process, my work could be used to design therapies aiming at controlling the equilibrium between these different populations in diverse pathologies such as cancer, viral infection, or intestinal diseases.