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Transcriptional Control of NK and iNKT Cell Development

Jiang Zhang

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Transcriptional Control of NK and iNKT Cell Development

**Contrôle Transcriptionnel du Développement des Cellules
NK et iNKT**

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Abstract

Natural killer (NK) cells are innate lymphoid cells widely recognized as important effectors during antiviral and anti-tumor responses. T-bet and Eomes are two transcription factors from the T-box family that are homologous with each other for protein sequence and DNA binding preferences. Both factors were previously shown to regulate NK cell development, but how they work together remains unclear. In this study, we identified complementary roles of Eomes and T-bet in the control of gene expression during NK cell maturation and found that T-bet and Eomes regulate mostly different gene sets and at different maturation stages. Analysis of genomic binding revealed a significant overlap between Eomes and T-bet. In addition, in silico analysis of DNA binding suggests that Eomes and T-bet rely on other co-factors to allow TF-specific activity. Moreover, T-bet or Eomes also regulate chromatin accessibility resulting in the control of NK cell development.

Invariant natural killer T (iNKT) cells are unconventional T cells bearing an invariant T cell receptor and are distinct from conventional CD4 or CD8 single positive T cells. Previous analyses suggested a role for Zeb1 in T cell development. We demonstrated that Zeb1 was essential for the development of NK1.1⁺ T cell especially iNKT cells. The truncated form of Zeb1 in the *Cellophane* mutant mouse abrogated iNKT cell development mainly through the deregulation of TCR signaling and survival and the repression of proliferation in T cell progenitors including DN2 and DP stages. A transcriptomic analysis on WT and *Cellophane* DP revealed that Zeb1 regulated the expression of multiple genes involved in cell cycle and TCR signaling, which was consistent with the phenotypes that we observed in mice. Finally, multiple lines of evidence suggest that Zeb1 acts in coordination with E-proteins such as TCF1 and HEB during T cell development.

Résumé

Les cellules tueuses naturelles (NK) sont des cellules lymphoïdes innées largement reconnues comme des effecteurs importants lors des réponses antivirales et anti-tumorales. T-bet et Eomes sont deux facteurs de transcription de la famille des T-box dont le domaine de liaison à l'ADN est très homologue. Il a été précédemment démontré que ces deux facteurs régulent le développement des cellules NK, mais la façon dont ils se coordonnent demeure mal connue. Dans cette étude, nous avons identifié des rôles complémentaires d'Eomes et de T-bet dans le contrôle de l'expression des gènes pendant la maturation des cellules NK et avons constaté que T-bet et Eomes régulent principalement différents groupes de gènes et à différents stades de maturation. L'analyse de la liaison génomique a révélé un chevauchement significatif entre Eomes et T-bet. De plus, l'analyse *in silico* de la liaison à l'ADN suggère qu'Eomes et T-bet dépendent d'autres co-facteurs pour exercer leur activité spécifique. De plus, T-bet et Eomes régulent l'accessibilité à la chromatine, ce qui entraîne le contrôle de la différenciation des cellules NK.

Les cellules T tueuses naturelles invariantes (iNKT) sont des cellules T non conventionnelles portant un récepteur de cellules T (TCR) invariant et sont distinctes des cellules T CD4 et CD8 conventionnelles. Des analyses précédentes ont suggéré un rôle pour Zeb1 dans le développement des cellules T. Nous avons démontré que Zeb1 était essentiel pour le développement des cellules T NK1.1⁺ en particulier les cellules iNKT. Dans les souris mutantes *Cellophane* une forme de Zeb1 tronquée est exprimée, ce qui entraîne un défaut de développement des cellules iNKT principalement par dérégulation de la signalisation du TCR et par une répression de la prolifération et de la survie dans les progéniteurs des lymphocytes T aux stades DN2 et DP. Une analyse transcriptomique sur WT et *Cellophane* DP a révélé que Zeb1 régulait l'expression de plusieurs gènes impliqués dans le cycle cellulaire et la signalisation TCR, ce qui était cohérent avec les phénotypes que nous avons observés chez la souris. Enfin, plusieurs éléments de preuve suggèrent que Zeb1 agit en coordination avec les protéines E telles que TCF1 et HEB pendant le développement des lymphocytes T.

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List of abbreviations

ADCC	Antigen-dependent cellular cytotoxicity
ATLL	Adult T-cell leukemia/lymphoma
BCR	B cell receptor
BM	Bone marrow
bZIP	Basic leucine zipper motif
CCR7	CC-chemokine receptor 7
CEACAM1	Carcinoembryonic-antigen-related cell-adhesion molecule
CHILP	Common helper-liker innate lymphoid cell progenitor
ChIP	Chromatin immunoprecipitation
CILP	Common innate lymphoid progenitor
CLP	Common lymphoid pregenitor
CMP	Common myeloid pregenitor
CMV	Cytomegalovirus
CtBP	C-terminal binding protein
CTCF	CCCTC-binding factor
CTCL	Cutaneous T cell lymphoma
CTL	Cytotoxic T lymphocyte
DBD	DNA-binding domain
DC	Dendritic cells
DNAM1	DNAX accessory molecule-1
DP	Double positive
E4bp4	E4-binding protein 4
ELP	Earliest lymphoid progenitor
EMT	Epithelial to mesenchymal transition
Eomes	Eomesodermin
ETP	Early T-cell-lineage progenitor
Ets	E26 transformation specific
GM-CSF	Granulocyte macrophage-colony stimulating factor
HCMV	Human cytomegalovirus
HD	Homeodomain

HSC	Hematopoietic stem cell
HSV-2	Herpes virus 2
ICAM-1	Intercellular adhesion molecule-1
Id	Inhibitor of DNA-binding
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
ILCP	Innate Lymphoid cells precursor
iNK	Immature natural killer cell
iNKT	invariant natural killer T cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
JAK	Janus kinase
KIR	Killer-cell immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor G1
LFA-1	Leukocyte function-associated antigen-1
LN	Lymph node
LTi	Lymphoid tissue-inducer cell
LTiPs	LTi precursors
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MIC	MHC class I chain related
MITF	Microphthalmia transcriptional factor
mNK	Mature natural killer cell
MyD88	Primary response protein 88
NCR	Natural cytotoxicity receptor
NFAT	Nuclear factor of activated T-cells
NK	Natural killer cell
NKP	Natural killer precursor
NKT	Natural killer T cell

PBMC	Periphery blood mononuclear cell
PTK	Protein tyrosine kinase
PU.1	Purine rich box-1
RAET1	Retinoic acid early transcript 1
S1PR5	Sphingosine-1 phosphate receptor 5
SAP	SLAM-associated protein
SH2	SRC homology 2
SHIP	SH2-domain-containing inositol-5-phosphatase
SLAM	Signaling lymphocyte activation molecule
SP	Single positive
STAT	Signal transducer and activation of transcription
Styk1	Serine/threonine/tyrosine kinase 1
TCR	T cell receptor
TF	Transcription factor
Th1	T helper 1 cell
TNF	Tumor-necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
Zeb	Zinc-finger E homeobox-binding
ZF	Zinc finger
β 2m	β 2-microglobulin
γ c	γ -chain

1 Natural Killer Cells

The immune system is classified into two categories: innate immunity and adaptive immunity. Innate immunity refers to rapid effector functions exerted by a variety of myeloid and lymphoid cells through a limited repertoire of germline-encoded receptors. In contrast, adaptive immune cells, including T and B cells, express a diverse repertoire of antigen receptors (TCRs and BCRs) that are produced by somatic recombination. Compared to innate immunity, adaptive immunity requires more time to develop, as T and B lymphocytes need to undergo division and maturation before they can exert their effector functions upon recognition of antigen ligands.

Natural killer (NK) cells are innate lymphocytes that differ from B and T cells in that they use numerous receptors, none of which is encoded by genes that undergo rearrangement (Murphy et al., 1987). NK cells were first discovered in 1975 and were originally characterized by their ability to lyse certain tumors without previous stimulation (Kieślinski et al., 1975; Herberman et al., 1975) (Figure 1). Moreover, the "missing-self hypothesis" was proposed by Klas Kärre afterwards declaring that NK cells used a different strategy than T cells to fight against pathogens and tumors (Kärre, 1985). NK cells principally participate in innate immunity but also contribute to regulating the outcome of adaptive immune responses. In contrast to the response of the adaptive immune system, the response of innate immune cells is immediate and creates the first line of protection during the first few days of infection with pathogens. NK cells are involved in the defense against viruses, intracellular bacteria and parasites, as well as in the elimination of tumors (Biron et al., 1989, 1999; Laskay et al., 1993; Yu et al., 1992).

1.1 Development

Lymphocytes originally derive from hematopoietic stem cells (HSCs) which can be found in many different sites during life, including yolk sac and liver in fetus, and bone marrow (BM), thymus, spleen, omentum and liver in adults. The BM microenvironment is a special niche providing cytokines, growth factors and

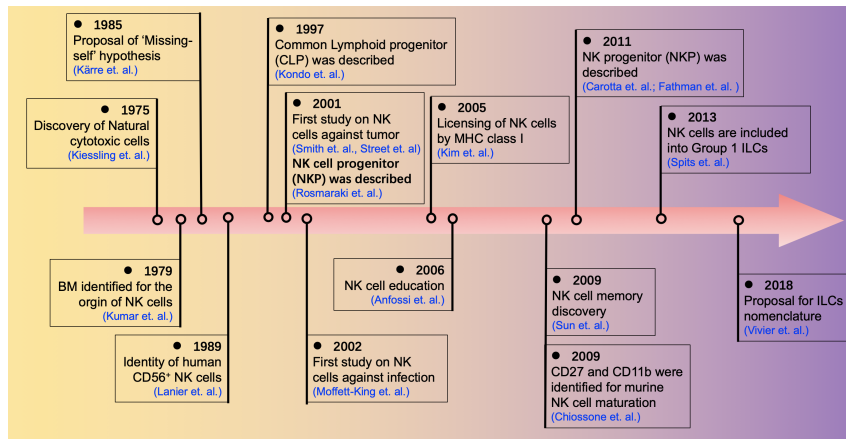


Figure 1. The timeline of NK cell discovery and research. NK cells were first discovered in 1975 by Kiessling et al. and the last two decades saw great advances in clarifying the identity and function of NK cells along with the arrival of new technologies including flow cytometry, Crispr-casp9 and high-throughput sequencing. The literature cited in the figure is listed in the Reference section.

extracellular matrix that support NK cell development and maturation. Of note, the acquisition of the CD122 (IL-15R β chain) is a critical point in NK cell differentiation as IL-15, constitutively produced by BM stroma cells, promotes NK cell differentiation, maturation and survival (Colpitts et al., 2013; Cui et al., 2014). Neither the thymus nor the spleen seem to be indispensable for the generation of NK cells as mice and humans deprived of thymus or spleen have normal NK cells in the peripheral blood in terms of numbers and functions (Sihvola and Hurme, 1984; Ramos et al., 1996; Schwarz and Hiserodt, 1990; Passlick et al., 1991). Both the fetal thymus and liver were traditionally recognized to contain hematopoietic precursors that can potentially differentiate into NK cells. However, NK cells from liver are reported to contain two populations, conventional NK cells, and tissue-resident NK cells which are described as a separate lineage that is dependent on T-bet but not Eomes (Peng et al., 2013; Daussy et al., 2014). The distribution of NK cell stages between early-life and adulthood is quite different (Collins et al., 2017). Further studies are still required to understand the biological importance of fetal NK cell development and the difference between fetal- and adult-derived NK cells in terms of lifespan and functions.

Several processes occur in the BM including generation of NK precursors (NKPs) via common innate lymphoid progenitors (CILPs) that are derived from common lymphoid progenitors (CLPs) (Yang et al., 2015b), acquisition of NK-cell inhibitory and activation receptors, NK cell education and functional competence. However, the BM is not the

only organ that contains NKPs and immature NK cells, as these cells can also be found in thymus, spleen, liver and lymph nodes (Vosshenrich et al., 2005; Freud et al., 2005; Takeda et al., 2005). This indicates that multiple sites might support NK cell differentiation.

In general, NK cell development can be divided into three stages (Colucci et al., 2003): an initial phase which involves hematopoietic stem cells that differentiate to the lymphoid lineage and then to the NKPs; a second phase involving the commitment of NKPs towards NK cells of phenotypic and functional characteristics; a final phase, which involves circulation and migration of NK cells to the peripheral organs (Figure 2).

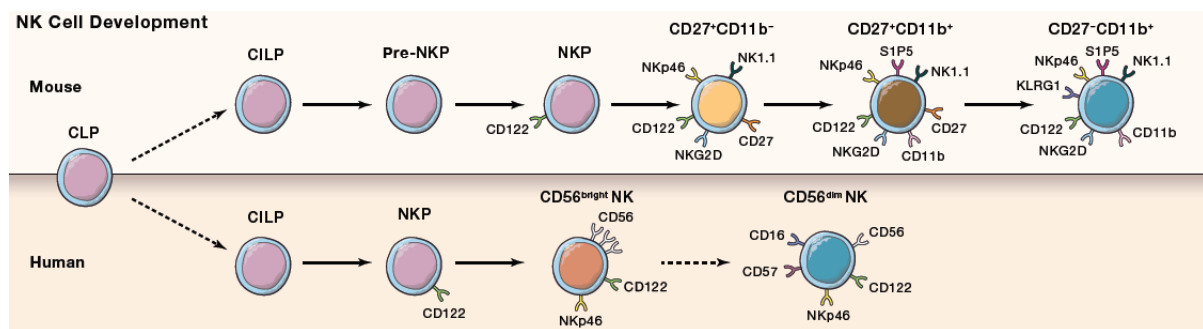


Figure 2. NK cell development and maturation. Mouse and human NK cells both derive from CLPs followed by CILPs which give rise to NK cells and ILCs. CD122 expression begins at the NKP stage. Commitment to the NK lineage is followed by NK1.1 or Nkp46 expression. However, mouse and human NK cells express different surface markers during maturation. In mice, NK cell maturation can be defined by CD11b and CD27 (i.e. CD11b⁻CD27⁺, CD11b⁺CD27⁺, CD11b⁺CD27⁻), while surface markers CD56 and CD16 allow the discrimination of two subsets of human NK cells, CD16⁺CD56^{dim} and CD16⁻CD56^{bright}. However, maturation is not a clear concept in human, although CD27^{low} NK cell subset is likely at a more mature stage in human NK cell differentiation based on the expression profile of NKR, other differentiation markers and functional maturity (Silva et al., 2008). Adapted from Crinier et al. (2020).

1.1.1 Commitment to becoming a killer

Within the hematopoietic system, multipotent, self-renewing hematopoietic stem cells (HSCs) provide the cellular substrate for the generation of different hematopoietic lineage precursors (e.g. erythroid, megakaryocytoid, myeloid and lymphoid cells). Hematopoiesis is a continuous process that is characterized by the progressive loss of cell-fate potentials. Firstly, HSCs differentiate into two major lineages: common

myeloid progenitors (CMPs ([Akashi et al., 2000](#)) and common lymphoid progenitors (CLPs) ([Kondo et al., 1997](#)). During lymphopoiesis, erythroid, megakaryocytoid and myeloid potentials are lost, resulting in the generation of CLPs that are restricted to the lymphoid fate. Dynamic regulation of transcriptional programs is involved in this commitment, with Ikaros, E2A, PU.1 and GFI1 (growth factor independent 1) strongly promoting lymphoid-specific program and repressing the myeloid-restricted program ([Ng et al., 2009](#)). Once generated, CLPs are immediately committed to the earliest lymphoid progenitors (ELPs) ([Igarashi et al., 2002](#)), and then further dedicated to give rise to B- and T-cell restricted precursors that are equipped with transcriptional programs leading to B cell or T cell fates ([Rothenberg, 2014](#)).

Two decades ago, researchers identified the earliest murine NK cell committed precursor residing within fetal thymus and adult bone marrow, that is Lin⁻ population (CD3⁻ CD19⁻ Gr1⁻ Ter119⁻) expressing IL-2R β (also known as CD122) ([Rosmaraki et al., 2001](#); [Ikawa et al., 1999](#)). These cells were later recognized as NK precursors (NKPs) and have the potential to generate NK cells but neither B/T cells, nor myeloid cells. In 2011, two different groups ([Carotta et al., 2011](#); [Fathman et al., 2011](#)) studied the potential of different progenitor populations to develop into NK cells. They identified several NK cell progenitor subsets including CD135⁺ CLP as expected and also two other populations that lacked CD135 expression and expressed variable levels of CD122. The CD122⁻ population was designated as pre-NKP, while the CD122⁺ subset probably corresponded to the previously defined NKP subset. The refined NKPs are lineage-negative, express variable CD122, CD127 and lack the expression of specific surface markers of NK cells such as NK1.1 in mouse and CD56 in human and inhibitory receptors ([Fathman et al., 2011](#)). Compared to well-equipped NK cells, NKPs are non-lytic and do not produce cytokines (e.g. interferon- γ). In general, CD122 expression can be defined as a characterized marker for NKPs denoting NK lineage-committed cells.

1.1.2 Making a mature killer

The transition from NKPs to NK cells is driven by several TFs (e.g. Nfil3/E4bp4) (Gascoyne et al., 2009) and cytokines (e.g. IL-15) (Kennedy et al., 2000; Barton et al., 1998), and during this process, NK cells acquire a characteristic cell-surface phenotype and the capacity to exert effector functions. The traditional way to identify NK cells by flow cytometry is to use CD3 to exclude T cells and to gate on cells positive for an NK cell-specific marker (NK1.1 epitope for C57BL/6 and C57BL/10 mice, CD49b or NKp46 for all mouse strains and CD56 for humans). Immature NK cells generated from NKPs start to express the C-type lectin NK-cell receptor protein 1 (NKR-P1) which is encoded by the *Nkrp1c* gene and recognized by the anti-NK1.1 antibody but only in certain mouse strains (Bennett et al., 1996; Rosmaraki et al., 2001). Although NK1.1 is widely used as a marker for NK cells in mice, it is not exclusive for NK cells as iNKT cells and some $\alpha\beta$ and $\gamma\delta$ T cell subsets also express NK1.1. In human, the NK cell definition is based on the expression of CD16 and CD56 while CD56 is also expressed by human IL-22-producing ILCs (Cella et al., 2009; Crellin et al., 2010). One of the natural cytotoxicity receptors, NKp46, seems to be a more specific and conserved NK cell marker in all mammalian species to detect NK cells. However, a small sub-population of T cells and IL-22-producing ILCs also express it (Walzer et al., 2007a). Moreover, our lab identified Serine/threonine/tyrosine kinase 1 (Styk1) as a marker of NK cells, but Styk1 is also expressed to some extent by other NK1.1 expressing cells such as liver type 1 innate lymphoid cells (ILC1s) and NK1.1⁺ $\gamma\delta$ T cells (Fauteux-Daniel et al., 2019). Collectively, the search for a specific pan-NK-cell marker might not be over, but a "perfect" marker might not exist.

NK cell maturation is a dynamic and continuous process in both mouse and human. In mouse, NK cells go through several stages of maturation. After acquisition of NK cell-specific markers NK1.1 and NKp46, NK cells acquire a full capacity of proliferation and start to sequentially express CD94/NKG2 receptors and Ly49 receptors and then further upregulate CD11b, CD43 and killer cell lectin-like receptor G1 (KLRG1). This process is associated with reduced proliferation and acquisition of effector functions (Kim et al., 2002). Distinct NK cell subsets can be identified via the

combination of costimulatory receptor CD27 and the integrin α_M (CD11b) (Chiossone et al., 2009). Adoptive transfer assay *in vivo* demonstrated that mouse NK cells went through four sequential development stages $CD11b^{low}CD27^{low}$ (DN) \rightarrow $CD11b^{low}CD27^{high}$ ($CD27^{low}$) \rightarrow $CD11b^{high}CD27^{high}$ (DP) \rightarrow $CD11b^{high}CD27^{low}$ ($CD27^{low}$) (Chiossone et al., 2009; Hayakawa and Smyth, 2006). $CD11b^{low}CD27^{low}$ (DN) cells were initially described as the most immature NK cells, but they were later found to be innate lymphoid cells group 1 cells (ILC1s) (Daussy et al., 2014). $CD11b^{low}$ cells are likely to proliferate at high rate while DP cells (rather than $CD27^{low}$ cells) are the most potent effector cells (Hayakawa and Smyth, 2006). $CD27^{low}$ cells express the highest level of Ly49 receptors among the three different populations and express the chemokine receptor CX3CR1 (Grégoire et al., 2007) and the sphingosine-1 phosphate receptor S1PR5 (Walzer et al., 2007b; Mayol et al., 2011) important for homing to peripheral organs.

In human, the development of NK cells can be stratified into five stages using a combination of antibodies against CD34, CD117, CD94, and CD16 among Lin^- cells (Figure 3) (Freud et al., 2014). Further study on human NK cell development in secondary lymphoid tissues divided stage 4 into two sub-populations according to the expression of C-type lectin-like surface-activating receptor, NKp80: $NKp80^-$ (stage 4a) and $NKp80^+$ (stage 4b). Transplantation assay into immunodeficient mice demonstrated that cells from stage 4a could give rise to stage 4b, and stage 5 cells sequentially (Freud et al., 2016). Interestingly, some CD57 and KIRs expression, which are associated with terminal differentiation among human PB NK cells (Lopez-Vergès et al., 2010; Björkström et al., 2010), were detected within stage 5, but not within stage 3–stage 4b populations. NK cells can be classified into 2 subsets considering the levels of CD56 and CD16 expression: $CD56^{bright}CD16^{lo/-}$ ($CD56^{bright}$) and $CD56^{dim}CD16^+$ ($CD56^{dim}$) cells. $CD56^{bright}$ cells are mostly found in lymphoid organs and express few inhibitory receptors (e.g. KIRs) but display robust proliferation and rapid production of immunomodulatory cytokines and chemokines in response to cytokine stimulation. By contrast, $CD56^{dim}$ cells are dominant in the peripheral blood and express high levels of activating and inhibitory receptors (e.g. CD94, KIRs), and develop potent cytotoxic functions (Yu et al., 1998; Sivori et al., 2002; Caligiuri, 2008). However, whether $CD56^{bright}$ cells are the immature

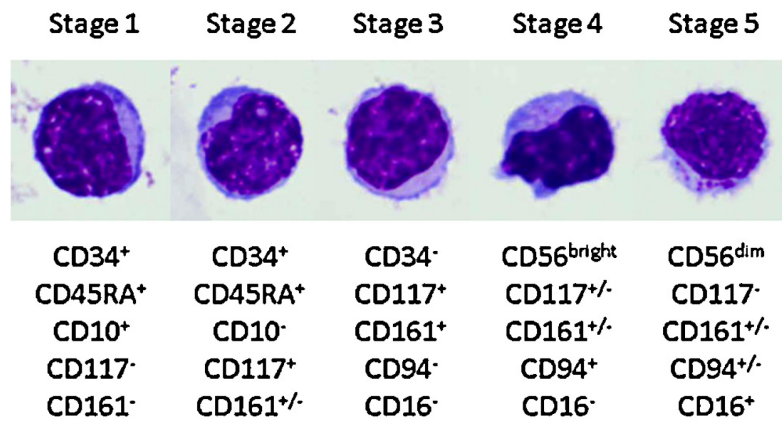


Figure 3. The stages of human NK cell maturation in secondary lymphoid tissues. NK cell developmental intermediates were viably purified from a pediatric tonsil followed by centrifugation of the cells onto glass slides and subsequent staining with Wright-Giemsa stain. Original magnification = 1000x. **Adapted from Freud et al. (2014).**

precursors of the CD56^{dim} is not definitely established (Michel et al., 2016).

In general, immature NK cells highly proliferate but fail to exert powerful response to target cells or produce interferon- γ (IFN- γ), while mature NK cells are less potent in proliferation but are equipped with the full repertoire of inhibitory receptors (Ly49 or KIR) which endows them with robust functions including cytotoxicity and cytokine secretion. In exceptional cases, mature NK cells can enter an expansion phase upon pathogen stimulation (Kim et al., 2002).

1.1.3 Dispatching killers to the frontline

It has been appreciated that NK cells are widely distributed in mammals both in lymphoid and non-lymphoid organs. In mouse, NK cell frequencies appear to be the highest in non-lymphoid organs such as liver and lung. The following organs are listed in order according to the frequencies of NK cells in lymphocytes: lung > liver > periphery blood mononuclear cells (PBMCs) > spleen > BM > lymph node (LN) > thymus, where NK cells are almost undetectable (Grégoire et al., 2007). The frequencies of NK cells in human tissues are somewhat different from those of mice: BM > Lung > Spleen > blood > gut > LN > tonsil (Dogra et al., 2020). The ratio between NK cell subsets defined by CD11b and CD27 in mice and CD56 in human also varies within tissues which is illustrated in details in Figure 4 (Crinier et al.,

2020).

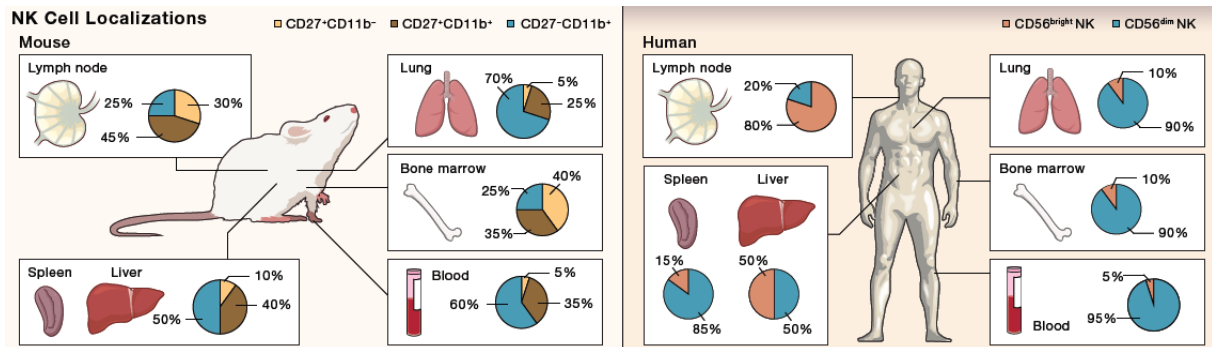


Figure 4. NK cell distribution. NK cells are localized in multiple sites including lymphoid and non-lymphoid organs (BM, liver, lung, spleen, LN, blood). The composition of NK cells from certain organs are individually shown in pie charts. In addition, NK cells are also present in other organs such as intestine, skin, tonsils, thymus, kidney, adipose tissue and pancreas. Adapted from [Crnier et al. \(2020\)](#).

Immature CD11b^{low} NK cells predominate in the BM and LN, while DP and mature CD27^{low} NK cells are present at higher frequencies in the blood, liver, spleen and lung. In humans, CD56^{dim} NK cells show stronger migration in response to chemokine CXCL8 (also known as IL-8) and soluble fractalkine (CX3CL1), whereas CD56^{bright} NK cells better respond through CC-chemokine receptor 7 (CCR7) which is associated with homing to lymph nodes. This results in 10 times more CD56^{bright} NK cells found in LNs than in the blood ([Frey et al., 1998](#)). CD56^{dim} cells comprises 5-20% of lymphocytes in blood and are essential for effector functions. Besides, NK cells can also be found in skin ([Ebert et al., 2006](#)) and placenta during pregnancy ([Moffett-King, 2002](#); [Freud et al., 2017](#)).

Taken together, NK cells patrol both lymphoid and non-lymphoid organs in the circulation and are ready to extravasate inflammatory tissues to offer protection, and the phenotype of NK cell subsets is not fixed but can evolve depending on the cellular context (signals from cytokines, chemokines, adhesion molecules...).

1.2 NK Cell Surface Receptors

NK cell activation is controlled via a dynamic balance between stimulatory and inhibitory receptors ([Vivier et al., 2008](#); [Gasser and Raulet, 2006](#); [Long, 1999](#)). Most

inhibitory receptors of NK cells are specific for MHC class I molecules that are expressed by normal cells but are often lost in infected cells or tumors to escape the attack from adaptive immunity (e.g. T and B cell response).

Ligands for activating receptors are usually poorly expressed by healthy cells but are upregulated by "unhealthy" cells, such as transformed, infected, or stressed cells ([Gasser and Raulet, 2006](#)). "Healthy" cells fail to be killed by NK cells as the inhibitory signaling counteracts with the activating signaling. In the context of "unhealthy" cells, increased expression of stimulatory ligands can overcome inhibitory signaling thus resulting in target cell lysis by NK cells ([Long, 1999](#)). Importantly, each NK cell receptor is often expressed only by a fraction of NK cells, independently of other receptors, so that the NK cell population is of great diversity due to the combinatorial expression of those receptors ([Raulet et al., 1997](#); [Valiante et al., 1997](#); [Kubota et al., 1999](#)). For examples, 10-15% of NK cells in C57BL/6 mice were shown to lack self-engaged inhibitory receptor (i.e. Ly49C, Ly49I and CD94-NKG2A) ([Fernandez et al., 2005](#)).

Engagement of activating receptor initiates either protein tyrosine kinase (PTK)-dependent pathways that rely on intracytoplasmic ITAMs (immunoreceptor tyrosine-based activation motifs), or the DAP10 transmembrane-dependent signaling adaptor-dependent pathway. On the contrary, inhibitory receptors can antagonize activating pathways through protein tyrosine phosphatases (PTPs) that are recruited via intracytoplasmic ITIMs (immunoreceptor tyrosine-based inhibition motifs) ([Vivier et al., 2004](#)). In addition to activating receptors and inhibitory receptors, NK cells also express many other surface receptors, such as cytokine, chemotactic and adhesion receptors ([Figure 5](#)).

1.2.1 Inhibitory receptors

"Missing-self"

The "missing-self" hypothesis was first put up forward by Klas Kärre in 1981 ([Kärre, 1981](#)) and officially published at the 1985 International Workshop on NK cells ([Kärre,](#)

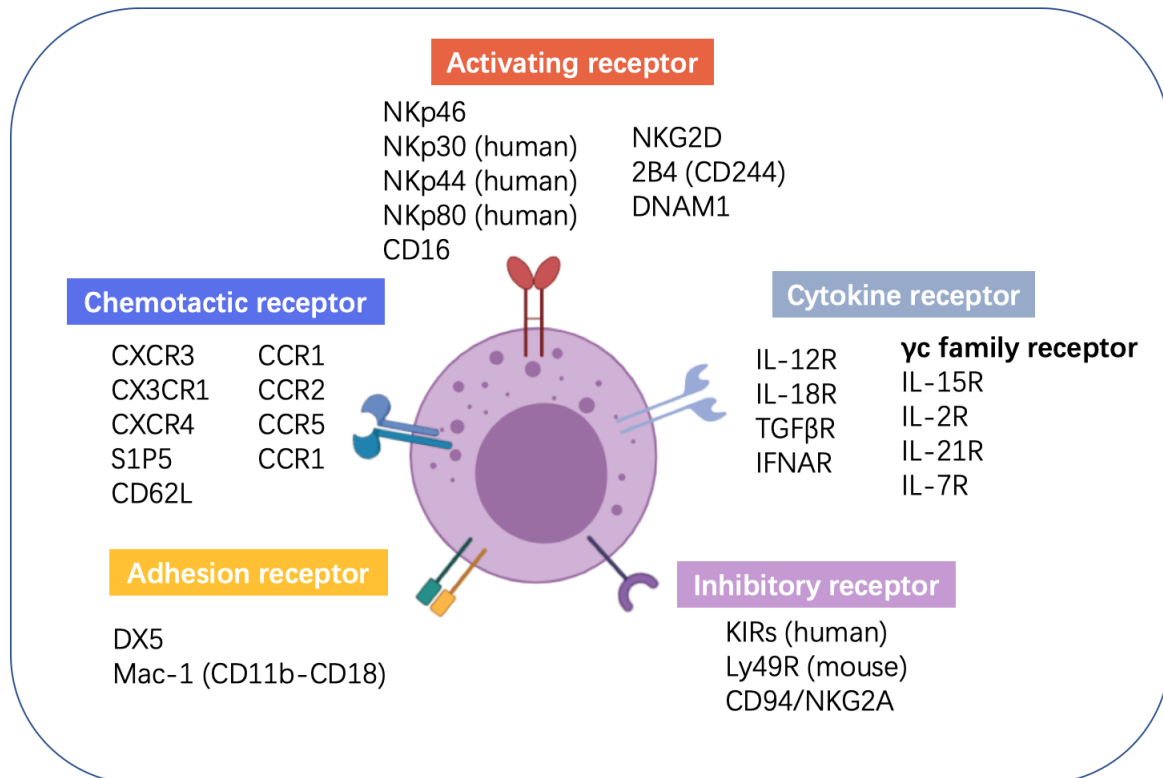


Figure 5. NK cell receptors. NK cells express a variety of receptors that can be classified into five groups: inhibitory receptors (purple), activating receptors (red), cytokine receptors (grey), chemotactic receptors (blue) and adhesion receptors (yellow). Only part of the receptors are listed here.

1985; Kärre et al., 1986) on the basis of results obtained in the field of F1 hybrid resistance and related allorecognition phenomena (Cudkowicz and Stimpfling, 1964b,a; Snell et al., 1967). By comparing NK and T cell reactivity, he found that in several situations (e.g. tumor versus healthy, virus infected versus uninfected), target cells sensitive to killing by NK cells would be resistant to killing by T cells, and vice versa. For example, some metastatic cells had lost MHC (major histocompatibility complex) expression presumably to escape T cell immunity, however, NK cells were shown to eliminate them in the circulation. Moreover, NK cell-resistant targets usually express more MHC class I molecules than their sensitive counterparts. This led him to propose the "missing-self" hypothesis that NK cells attack cells lacking self-MHC, which could be a "backup" surveillance system to eliminate target cells without MHC (Kärre, 2008).

The working model was formally demonstrated later when Kärre showed in mouse experiments that tumors developed only in mice inoculated with MHC-I⁺ tumors but

not with MHC I-deficient ones (Kärre et al., 1986). Upon NK cell depletion with the monoclonal anti-NK1.1 antibody, MHC I negative tumors also grew in recipient animals, thus demonstrating that NK cells were the critical effector cells in this model (Ljunggren and Kärre, 1985). Similar results were observed in nude mice further excluding the possibility that NKT cells were involved in this process. Thus, one of the most important NK cell functions is to detect and eliminate cells that fail to express normal self markers, resulting in a completely opposite action of NK and T cells. However, both NK and T cells are strongly influenced by the target MHC molecules.

NK cells have a variety of MHC class I-specific inhibitory receptors including killer cell immunoglobulin-like receptors (KIRs) in human, lectin-like Ly49 molecules in mice, and CD94/NKG2A heterodimers in both species (Figure 6) (Karlhofer et al., 1992). These

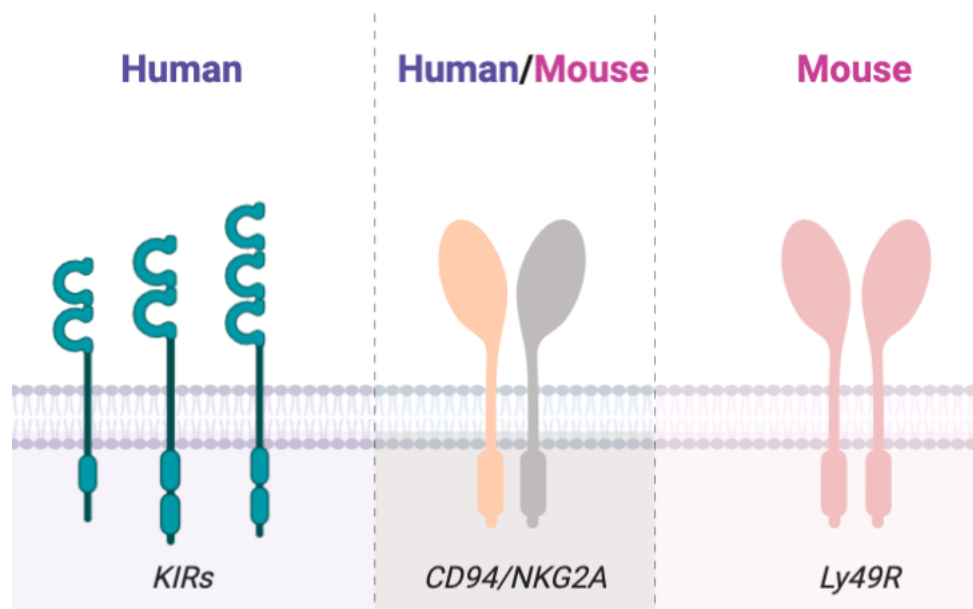


Figure 6. Inhibitory NK cell receptors in mouse and human. Human and mouse NK cell Intracytoplasmic ITIM-bearing receptors are depicted. KIRs have a long cytoplasmic tail containing ITIM and two or three extracellular Ig-like domains, conferring their specificity for HLA ligands. CD94/NKG2A are shared between human and mouse, while Ly49R is exclusively expressed by mouse NK cells.

inhibitory receptors contain cytoplasmic ITIMs (Colonna, 1997; Lanier, 1998; Moretta et al., 2000; Raulet et al., 2001). Upon ligand binding and subsequent tyrosine phosphorylation, ITIMs recruit SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatases such as SHP1 and SHP2 or SH2-domain-containing inositol-5-phosphatase (SHIP) to inhibit NK cell effector responses mediated through their activating receptors (Olcese et al., 1996). Apart from the MHC class I specific

receptors, some inhibitory receptors are described to bind to MHC-I-like molecules (e.g. NKG2A binds HLA-E in human or Qa1 in mice) or non-MHC-class-I molecules, among which are NKR-P1B and NKR-P1D ([Iizuka et al., 2003](#); [Carlyle et al., 2004](#)), KLRG1 ([Carlyle et al., 2004](#)), carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1) ([Markel et al., 2004](#)) in humans, and some of the Ly49 family members in mice.

The ability of NK cells to control MHC I negative cells has at least two benefits. In one hand, NK cells spare healthy cells that express self-MHC class I molecules and low amount of agonist and, on the other hand, NK cells are engaged in surveillance of virus-infected cells or tumors that downregulate MHC class I expression so as to avoid recognition by CD8⁺ cytotoxic T lymphocytes ([Anfossi et al., 2006](#); [Cooley et al., 2007](#)). Inhibitory receptors also confer NK cells the ability to maintain a state of responsiveness to subsequent activation events, a process named as education, as detailed later ([Kim et al., 2005](#)). In addition, co-inhibitory receptors including PD-1, Lag3, Tim-3 and TIGIT are proven to have important role in the maintenance of immune homeostasis and in regulating autoimmune diseases. More recently, the function of co-inhibitory receptors in NK cells has been manipulated to control the process of cancer highlighting the potential therapeutic approaches of targeting NK cells in the treatment of cancer ([Zhang et al., 2018b](#); [André et al., 2018](#); [Morvan and Lanier, 2016](#)).

1.2.2 Activating receptors

The "missing-self" model shows the existence and importance of inhibitory receptors on NK cells, but does not provide any explanation on how NK cells become activated when they encounter tumor or virus-infected cells, or "unhealthy" cells that lack MHC class I molecules.

Unlike T and B cells which possess a single dominant antigen receptor, NK cell activation relies on a vast combination of receptors to initiate effector functions. Long and colleagues argued that none of the receptors alone, exception of CD16, were able to trigger cytolytic activity or cytokine secretion upon cross-linking with agonistic

antibodies in freshly isolated primary NK cells (Bryceson et al., 2006). Rather than forming a hierarchy or dominance, activating receptors can cooperate to mediate cytokine secretion and natural cytotoxicity. CD16 is an exception, as it is sufficient to initiate degranulation by itself (Lanier et al., 1989, 1991). Redundancy may occur within and among synergistic combinations of receptors, allowing NK cells to gain a greater flexibility in sensing and responding quickly to changes in the environment (Long et al., 2013).

Numerous activating receptors are expressed in NK cells. Biochemically, they can be classified into three different categories based on signaling pathways: the ITAM-bearing NK receptor complexes (e.g. NKp46, CD94/NKG2C, CD16), the DAP10-associated NKG2D receptor complexes and the SAP/EAT-2 pathway (e.g. SLAM family receptor 2B4) (Lanier, 2008). However, there are other activating receptors associated with tyrosine signaling for which only limited studies about the downstream signaling have been conducted.

Receptors associated with ITAM-bearing molecules

The ITAM motif has been studied in great details as it supports the major signaling pathway downstream of most immunoreceptors, such as the T cell receptors (Smith-Garvin et al., 2009). Three ITAM-bearing molecules — FcR γ , CD3 ζ and DAP12 — are constitutively expressed in NK cells and contribute to signaling by engaging different activation receptors as either disulfide-bonded homodimers or, in the case of FcR γ and CD3- ζ , as disulfide-bonded heterodimers. All three ITAM-bearing molecules have minimal extracellular regions comprising only a few amino acids (principally the cysteine residues) through which they dimerize. FcR γ and DAP12 have a single ITAM, while CD3- ζ has three ITAMs per chain (Lanier et al., 1998). The ITAM-bearing proteins contain an aspartate charged residue, located centrally within the transmembrane of DAP12 and closer to the extracellular region in FcR- γ and CD3- ζ . The aspartate charged residue is required for stable association with the activating receptors, which typically contain an oppositely charged amino acid (lysine or arginine) (Lanier, 2008). Once these activating receptors bind to specific ligands, it will induce the phosphorylation of the ITAM tyrosines, presumably by Src family kinases, including Lck, Fyn, Yes, Lyn and Fgr. ITAM phosphorylation will

provide a docking site for Src homology (SH2) domains of tyrosine kinases Syk and ZAP70 (Smith-Garvin et al., 2009; McVicar et al., 1998; Colucci et al., 2002; Chiesa et al., 2006).

NKG2D (CD314)

NKG2D is a lectin-like type II transmembrane homodimer (Houchins et al., 1991) and is expressed on virtually all NK cells and various T cells subsets, including CD8⁺ T cells, $\gamma\delta$ T cells and NKT cells (Bauer et al., 1999; Jamieson et al., 2004). In humans, CD4⁺ T cells rapidly upregulated the expression of NKG2D after activation (Sáez-Borderías et al., 2006). However, NKG2D is not present in murine CD4⁺ T cells even after activation *in vitro* (Groh et al., 2006). The ligands for NKG2D are of great diversity, all of which are related self proteins that are similar to MHC class I molecules (Raulet and Guerra, 2009).

NKG2D signals through its association with the short transmembrane molecule DAP10, which contains the cysteine residues required for dimer formation and carries a tyrosine-based motif (YxxM) different from the ITAM. It is well documented that NKG2D signaling also participates in the elimination of tumor cells that express NKG2D ligands both *in vitro* (Bauer et al., 1999; Jamieson et al., 2004; Pende et al., 2001; Bryceson et al., 2006) and *in vivo* in tumor transplant experiments (Diefenbach et al., 2001; Cerwenka et al., 2001). In humans, specific genetic polymorphisms of NKG2D have been reported to be associated with susceptibility to cancer (Hayashi et al., 2006). Besides, infection with cytomegalovirus (CMV) leads to the upregulation of NKG2D ligand expression in mouse which indicates that NKG2D is also involved in protection from virus infections (Welte et al., 2003). However, enhanced resistance of NKG2D-deficient mice to MCMV infection was observed which might be a consequence of NK cell dysregulation resulting in higher frequency of NK cells capable and more sensitive to respond (Zafirova et al., 2009).

Receptors of the SLAM family

2B4 (also named CD244) is a member of the SLAM (signaling lymphocyte activation molecules)-related family, which mediates cell-cell interactions through homophilic binding and is expressed by all NK cells, $\gamma\delta$ T cells and memory CD8⁺ ($\alpha\beta$) T cells (Veillette, 2006). The SLAM family receptors transmit activation signals through

interaction between SH2 domain-containing adaptor proteins and the tyrosine-based motif TIYXX (V/I) (as an immunoreceptor tyrosine-based switch motif, ITSM) in the cytoplasmic domain of CD244. Three adaptor protein — SAP (SLAM-associated protein), EAT-2 and ERT — can recognize this motif (Veillette, 2006; Veillette et al., 2007).

Interestingly, both human and mouse 2B4 have been reported to be as either activating or inhibitory receptor, largely depending on the expression level of 2B4 and the degree of association with certain adaptor molecules (Schatzle et al., 1999; Lee et al., 2004; Chlewicki et al., 2008). Veillette and colleagues proposed that CD244-SAP-Fyn receptor complex activates NK cells, whereas CD244-EAT2 and CD244-ERT receptor complexes inhibit NK cell activation (Veillette, 2006; Veillette et al., 2007). The mechanism of the balance between activating and inhibitory functions of 2B4 remains not well understood.

DNAM1

DNAX accessory molecule-1 (DNAM-1, CD226) is an adhesion and costimulatory molecule that is constitutively expressed by most NK cells, T cells, macrophages and dendritic cells. DNAM-1 is known to promote NK cells cytotoxic activity and IFN- γ production upon binding to its ligands (Chan et al., 2010; Carlsten et al., 2007). DNAM-1 binds to two major ligands including the poliovirus receptor CD155 (also known as PVR) and the nectin adhesion molecule CD112 (known as PVRL2), both of which are upregulated on tumor cells (Soriani et al., 2009; Bottino et al., 2003). Herein, these ligands can activate or enhance tumor cells lysis, reject certain tumor cells and limit the formation of tumors *in vivo* (Gilfillan et al., 2008). Of note, DNAM-1 shares its ligands with the inhibitory molecules TIGIT and CD96. As a consequence, TIGIT reportedly counterbalanced CD226, whereas CD96 competed with DNAM-1 for CD155 binding and limited NK cell function by direct inhibition (Chan et al., 2014). Upon engagement, DNAM-1 Ser³²⁹ is phosphorylated by protein kinase C. Leucocyte function-associated antigen-1 (LFA-1) then cross-links with DNAM-1, resulting in the tyrosine phosphorylation of DNAM-1 which is induced by Fyn protein tyrosine kinase (Shibuya et al., 1999, 2003; Enqvist et al., 2015).

NCRs

A number of natural cytotoxicity receptors (NCRs) are also expressed on NK cells such as NKp46/NCR1, NKp30/NCR3 and NKp44/NCR2, among which only NKp46 is expressed in mouse. NKp46 and NKp30 combine with FcR γ and/or CD3- ζ , whereas NKp44 associates with the signaling adaptor DAP12 (Moretta and Moretta, 2004). NKp46 is one of the NCRs expressed on all NK cells (Pessino et al., 1998), which was recently shown to control TRAIL protein expression in both NK cells and ILCs (Sheppard et al., 2018). Numerous different NCR ligands have been identified, including heparan sulphate proteoglycans (for NKp30 and NKp46 (Bloushtain et al., 2004), viral hemagglutinins derived from influenza and parainfluenza (for NKp44 and NKp46) (Mandelboim et al., 2001), and the nuclear factor HLA-B-associated transcript 3 and B7-H6 (for NKp30) (von Strandmann et al., 2007; Brandt et al., 2009). It is demonstrated by a series of studies that the different DAP12-, FcR γ - and CD3 ζ -associated NK receptor complexes could use different adapters or kinases in NK cells depending on different stages of maturation or activation (Chiesa et al., 2006).

1.2.3 Cytokine receptors

The local environment has a great influence on NK cell homeostasis, differentiation and functions. NK cells express a series of cytokine receptors (Marçais et al., 2013) including γ c family receptors which share a common gamma chain (e.g. IL-15R, IL-2R, IL-21R, IL-7R, IL-9R and IL-4R) and IL-12 family receptors (e.g. IL-12R), IL-18R, TGF β receptor, IFNAR (Figure 7). Most cytokines transduce signals via Janus kinase / signal transducer and activator of transcription (JAK/STAT) pathways that contribute to the regulation of differentiation, proliferation, migration or functions (Stabile et al., 2018; Villarino et al., 2017). The JAK/STAT pathway is highly conserved among species, and includes four members of the JAK family (JAK1-3 and TYK2) and seven STAT proteins (STAT1-4, STAT5A, STAT5B, STAT6) (Gotthardt et al., 2019).

IL-15R is composed of three subunits: IL-15R α chain, β and γ chain. IL-15R and IL-2R share IL-2/15R β chain (CD122) and the common gamma chain (γ c, CD132), which is also a part of other receptors for IL-7, IL-4, IL21. The only difference between IL-2R and IL-15R lies in their α chain, IL-2R α (CD25) for IL-2R and IL-15R α



Figure 7. Schematic of essential cytokines in NK cell biology. IL-15R, IL-2R, IL-21R belong to the γ c receptor family. Engagement of related interleukin results in recruitment of associated JAK-STAT proteins. STAT proteins predominantly activated by relative cytokines are indicated. IL-18R is an exception as it employs MyD88 and leads to the activation of NK- κ B and MAPK pathway.

for IL-15R (Giri et al., 1995). Interestingly, IL-15 and IL-15R α are co-expressed by the same cells (e.g. dendritic cells and macrophages) and form a complex which can be trans-presented to neighboring cells expressing IL-15R β / γ c (Dubois et al., 2002). IL-15 signaling is shown to be a rate-limiting factor for both NK cell development and survival, and is also necessary for IFN- γ expression when coupled with IL-12. The absolute number of NK cells is reduced by roughly half in *IL-15*^{+/-} mice. Moreover, mice with transgenic overexpression of IL-15 showed early expansion of NK cells (Fehniger et al., 2001). Deletion of genes encoding Stat5b or both Stat5a and Stat5b, which are crucial mediators of IL-15 receptor signaling, recapitulates the phenotype found in IL-15 deficient mice in terms of NK cell development (Imada et al., 1998; Moriggi et al., 1999). ChIP-seq data revealed that STAT5 can directly bind to numerous genes that are required for cell proliferation, cell cycle and viability, which partly explains the need for IL-15 during NK cell development (Lin et al., 2012). Moreover, resting NK cells need to be primed before gaining their full effector capacity, and this process is also controlled by IL-15 produced by dendritic cells (DCs), or monocytes (Lucas et al., 2007; Mortier et al., 2009). A recent study from our team also demonstrated that high doses of IL-15 were required to activate the metabolic checkpoint kinase mTOR while exposure to low dose of IL-15 only resulted

in sustained phosphorylation of Stat5 without involvement of mTOR signaling (Marçais et al., 2014). Surprisingly, the few NK cells found in IL-15-deficient mice appeared relatively mature and expanded after one week of mouse CMV infection, and were capable of cytokine secretion and cytolytic function proposing a IL-15-independent NK cell response to infection (Vosshenrich et al., 2005; Sun et al., 2009b).

IL-2R signaling pathway is similar to that of IL-15R. IL-2 recapitulates most IL-15 functions in NK cells. However, unlike IL-15, IL-2 is not indispensable for NK cell homeostasis but is fundamental for NK cell priming against inflammatory stimulation (Vosshenrich et al., 2005). The presence of IL-2R α makes NK cells more sensitive to IL-15 and several studies showed that IL-2 is non-redundant during priming (Ring et al., 2012).

IL-21R is homolog to IL-2R β and its ligand IL-21 shows great similarity with IL-15. IL-21 binds to the common γ chain like IL-15R and IL-2R and signals through JAK1 and STAT5. IL-21 treatment can induce the expression of perforin and IFN- γ in NK cells thereby increasing their cytotoxicity and cytokine production (Brady et al., 2004). IL-21 was reported to inhibit NK cell proliferation mediated by IL-15 but to promote NK cell effector functions against viral infection. However, mice lacking IL-21R do not display any defect on NK cells in terms of numbers or maturation arguing against an essential role of IL-21 in NK cell biology under homeostatic conditions (Kasaian et al., 2002).

IL-12R binds to the heterodimeric cytokine IL-12 which is composed of two subunits IL-12p40 and p35. IL-12 is mainly produced by DCs and macrophages early after pathogen infection and detection through innate sensors (Goldszmid et al., 2012). The crosstalk between NK cells and DCs usually involves IL-12 delivery toward NK cells. The major role of IL-12 *in vivo* is to induce IFN- γ production as in IL-12p35-deficient mice, IFN- γ production was dramatically decreased while cytotoxicity remained unchanged during virus infection (Nguyen et al., 2002). IL-12 can synergize with several cytokines especially IL-18 to promote the IFN- γ secretion by NK cells. IL-12 activates STAT4 through combination of JAK2 and TYK2 (Figure 7) and to a lesser degree activates STAT1, STAT3 and STAT5 (Strobl et al., 2011). Moreover, it was shown that in T cells STAT4 could cooperate with NF- κ B at multiple

cis elements to promote IFN- γ expression ([Balasubramani et al., 2010](#)).

IL-18R belongs to the IL-1R family, which comprises 11 members. Upon IL-18 binding, IL-18R1 dimerizes with IL-18R accessory protein (IL-18RAP), which recruits myeloid differentiation primary response protein 88 (MyD88) and leads to the activation of NF- κ B and MAPK pathway. The main effect of IL-18 on NK cells is to couple with IL-12 to induce IFN- γ . Engagement of IL-12 signaling leads to STAT4 phosphorylation which transactivates IFN- γ transcription. The binding of IL-18 to its receptors stabilizes the IFN- γ mRNA and enhances IFN- γ production through activation of the MAPK pathway ([Marçais et al., 2013](#)).

1.2.4 Chemotactic receptors

NK cells are widely distributed throughout the body with a relatively high frequency in both lymphoid and non-lymphoid organs, such as BM, spleen, liver, lung, thymus, LN and uterus. Notably, the distribution of NK cells is dynamic and is governed by a network of chemokine receptors and other adhesion molecules (e.g. integrins and selectins) ([Figure 8](#)).

At steady state, S1PR5 and CXCR4 are of great importance for NK cells to egress from BM to reach the blood. CXCR4 retains NK cells in the BM whereas S1PR5 promotes their trafficking to the blood. S1PR5 expression increases during NK cell differentiation, while CXCR4 displays an opposite expression profile, with highest expression in immature NK cells but a moderate level in mature NK cells ([Walzer et al., 2007b](#); [Broxmeyer et al., 2005](#)). NK cell-expressed S1PR5 acts as a chemotactic receptor for S1P of which natural gradient promotes NK cells circulating in the blood, and homing to the spleen and lung. On the contrary, the high level of CXCR4 ligand CXCL12 found in local environments desensitizes CXCR4 in the BM. *In vivo* administration of CXCR4 antagonist releases NK cells from the BM showing that CXCR4 is essential for the retention of NK cells in the BM ([Mayol et al., 2011](#); [Broxmeyer et al., 2005](#)). By taking advantage of two transgenic mice, our team demonstrated that NK cell trafficking requires both CXCR4 desensitization and S1PR5 engagement while the two signals are independent of each other ([Mayol](#)

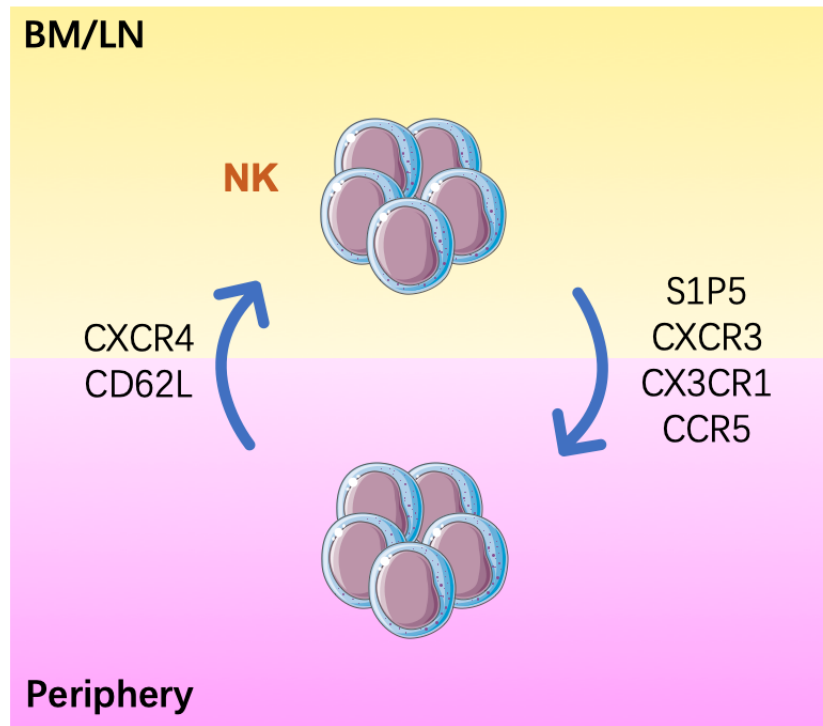


Figure 8. NK cell trafficking at steady state and inflammatory condition. NK cells mainly develop in the BM and are widely distributed in peripheral organs including spleen, liver, lung and uterus. The migration from BM to the periphery is governed by a series of chemotactic receptors and other adhesion receptors as indicated in this figure. Upon maturation, NK cells acquire expression of S1PR5 and exit the BM through a S1PR5-dependent way. This process can be also promoted by other chemokines like CXCR3, and CCR5 in inflammatory condition. CXCR4 and CD62L expression favor NK cells homing to the BM. The balance of chemokine receptor expression is important for the homeostasis of NK cells.

et al., 2011).

In the context of tumors, *CXCR3*^{-/-} mice had a dramatic reduction in tumor-infiltrating NK cells (especially DP population) indicating the importance of CXCR3 in NK cell recruitment under inflammatory conditions (Wendel et al., 2008). CXCR3 is expressed at higher level in murine DP subset and human CD56^{bright} NK cells while CX3CR1 is upregulated in murine CD27^{low}, and human CD56^{bright} cells. In the absence of CX3CR1, mature NK cells accumulated in the BM parenchyma due to abrogation of the early trafficking from BM (Ponzetta et al., 2013), but CX3CR1 deficient mice have normal peripheral NK cell numbers (TW, unpublished). Under stress or danger, NK cells are rapidly recruited to sites of inflammation as they express multiple inflammatory chemokine receptors (CCR5, CXCR3 and CX3CR1). A massive recruitment of NK cells is observed in the draining LN when stimulated by

TLR7/8 ligands or LPS-activated DCs in a CXCR3-dependent manner in mouse (Martín-Fontecha et al., 2004).

CD62L is essential for NK cells homing and recruitment to LN from spleen through interaction with glycosylated L-selectin ligands on high endothelial sinus (HEVs) (Chen et al., 2005). Moreover, expression of integrins enables NK cells to interact firmly with the vascular endothelium, like LFA1 (leukocyte function-associated antigen-1) (Bianchi et al., 1993). In human, it was also shown that CCR7 is upregulated on CD56^{dim} cells by IL-18 *in vitro* indicating that under inflammatory conditions, NK cells can also migrate to LN by modulating the level of CCR7 (Mailliard et al., 2005).

1.2.5 Adhesion receptors

Upon activation through NK cell activating receptors, NK cells first adhere to target cells through adhesion receptors followed by polarization of the actin cytoskeleton. Subsequently, cytotoxicity is completed when the content of cytotoxic granules is released towards target cells.

LFA-1 has been shown to be essential for NK cells binding to target cells. Deletion of LFA-1 on NK cells abrogates binding to its ligand intercellular adhesion molecule-1 (ICAM-1) and impairs cytotoxicity by preventing the formation of immunological synapse between NK cells and targets (Matsumoto et al., 1998; Barber et al., 2004). In addition, NK cells also express a variety of other integrins, including β_2 integrin family such as Mac-1 (also known as $\alpha_M\beta_2$ or CD11b-CD18) and DX5 (also known as $\alpha_2\beta_1$) (Lanier et al., 1986; Kim et al., 2002).

1.3 NK Cell Education

NK cells develop a sophisticated mechanism known as NK cell education to ensure functional competence and adaptation to the host cells. This process occurs through the recognition of self-MHC molecules by inhibitory receptors (Kim et al., 2005; Raulet and Vance, 2006; Anfossi et al., 2006; Elliott and Yokoyama, 2011). Instead of

being autoreactive, a subset of NK cells lacking inhibitory MHC I-specific receptors acquires a state of "hyporesponsiveness" to MHC-I-deficient targets or antibody-mediated engagement of activating signals (Fernandez et al., 2005). It is also worth noting that NK cells from either self-ligand deficient mice (e.g. $\beta 2m^{-/-}$ mice, $TAP^{-/-}$ mice and $H2-K^bD^b$ KO mice) or inhibitory receptor-deficient mice (e.g. NKC^{KD} mice) fail to reject non-self cells and poorly respond to stress (Bélanger et al., 2012), further proving that only NK cells that receive inhibitory signals during development can acquire full functional competence. Yokoyama and colleagues refer to this education process as "licensing" (Yokoyama and Kim, 2006), whereas Raulet and his colleagues avoid the licensing terminology but favor a "disarming" model arguing that the term licensing usually denotes an active mechanism in which functionality is conferred to a subject (Raulet and Vance, 2006). Both theories favor the education system developed by NK cells to maintain cell homeostasis in resting situation and to prepare them to be armed upon stimulation.

Several studies showed that NK cells need intact ITIMs signaling to be educated or respond to stimuli. Blocking ITIM signaling by deletion of SHP1 alters the expression of Ly49 receptors and impairs NK cell effector functions (Viant et al., 2014; Lowin-Kropf et al., 2000), which provides strong evidence to support the "arming" model (Kim et al., 2005). This "arming" model (Figure 9) proposed by Raulet and his colleagues postulates that self-MHC I-specific receptors provide all the signals required for licensing to occur (Raulet and Vance, 2006). To become functional, NK cells require the engagement of particular inhibitory receptor with self-MHC. When their activation receptors are crosslinked, they readily produce cytokines.

As is widely recognized, NK cell activation is regulated by both inhibitory and activating receptors. In general, engagement of activating receptors will allow NK cells to detect and kill transformed host cells or tumors. However, several studies performed *in vitro* or with tumor ligands *in vivo* showed that sustained engagement of activating ligands induce hyporesponsiveness in NK cells as well. Coupled with experiment on transgenic mice (e.g. constitutive Rae-1 expression), it clearly demonstrated that the sustained engagement of activating receptors exerts negative effects on NK cells (Oppenheim et al., 2005; Tripathy et al., 2008; Fauriat et al.,

2010a). Herein, NK cells experiencing continuous activation in the absence of inhibitory signals, become hyporesponsive or “disarmed”, which is known as the “disarming” model (Figure 9) (Raulet and Vance, 2006).

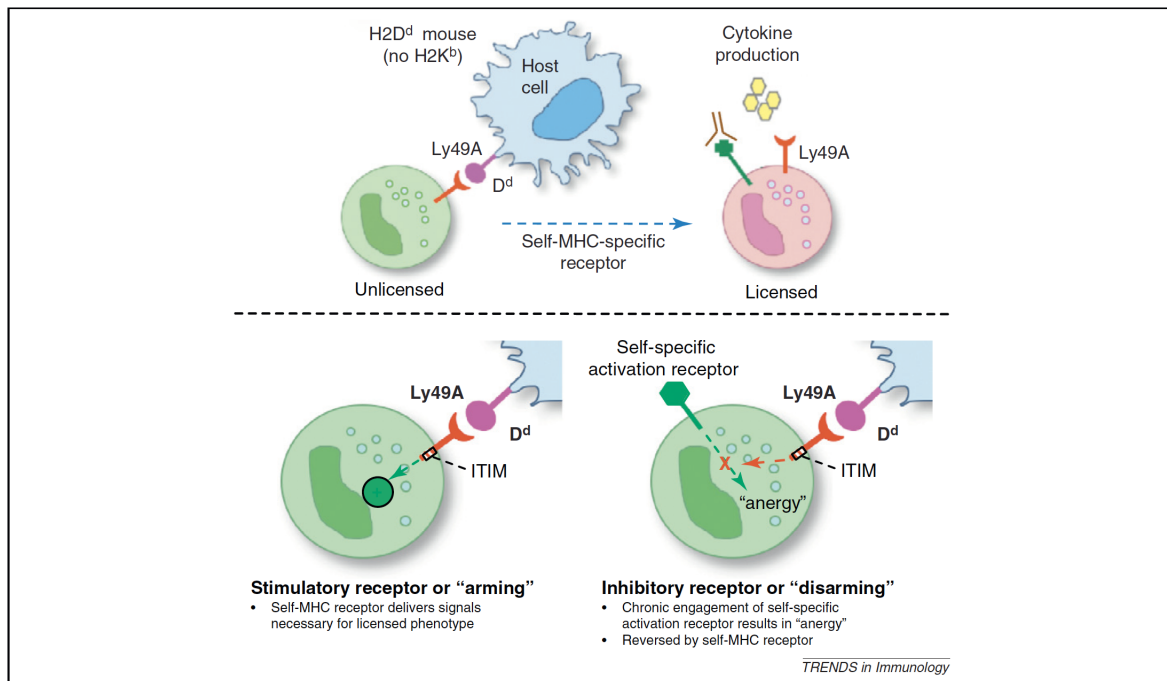


Figure 9. Arming versus disarming models. Depicted upper side is the situation in a mouse expressing only H2D^d, which, for the sake of simplicity in this diagram, can only be recognized by Ly49A and not by Ly49C. Only cells expressing Ly49A engage H2D^d as self-MHC, which results in licensed NK cells. When their activation receptors are crosslinked, they readily produce cytokines. Depicted lower side are the two major mechanisms, arming and disarming, that have been proposed to explain how the self-MHC-specific receptor confers licensing upon contact with self-MHC. NK cells require the engagement of self-MHC receptor signaling that is necessary for licensed phenotype. However, chronic engagement of self-specific activation receptor results in “anergy”, a situation that can be reversed by inhibitory signals. **Adapted from Elliott and Yokoyama (2011).**

However, uneducated NK cells can be reset to be responsive under tumor and virus infection (Fernandez et al., 2005; Orr and Lanier, 2011). In the context of murine cytomegalovirus (MCMV) infection, it is the uneducated NK cells rather than the educated ones that dominate the effector function. Following NKG2D ligation, non-educated Ly49-deficient NK cells were able to lyse RMA-S-Rae-1 tumor cells as efficiently as educated NK cells (Bélanger et al., 2012). Therefore, NK cell reactivity is a plastic process and not only depends on education but also on other signals such as cytokines produced during infections.

1.4 NK Cell Function

NK cells principally participate in innate immunity but also modulate adaptive immune responses. NK cells were first reported to be essential against early tumor development, as demonstrated by deleting NK cells using anti-NK1.1. They possibly collaborate with invariant NKT cells in the control of tumors (Smyth et al., 2001; Street et al., 2001). Over the years, studies showed that NK cells also participate in host response to pathogens and virus through cytokine and chemokine secretion. They are also important for maternal tolerance of the fetus as NK cells are also found in maternal-fetal interface (Moffett-King, 2002). All in all, two main functional activities can be associated with NK cells: (1) cytotoxicity and (2) capacity to immediately produce cytokines (e.g. tumor-necrosis factor (TNF) and IFN- γ) (Trinchieri, 1989) (Figure 10).

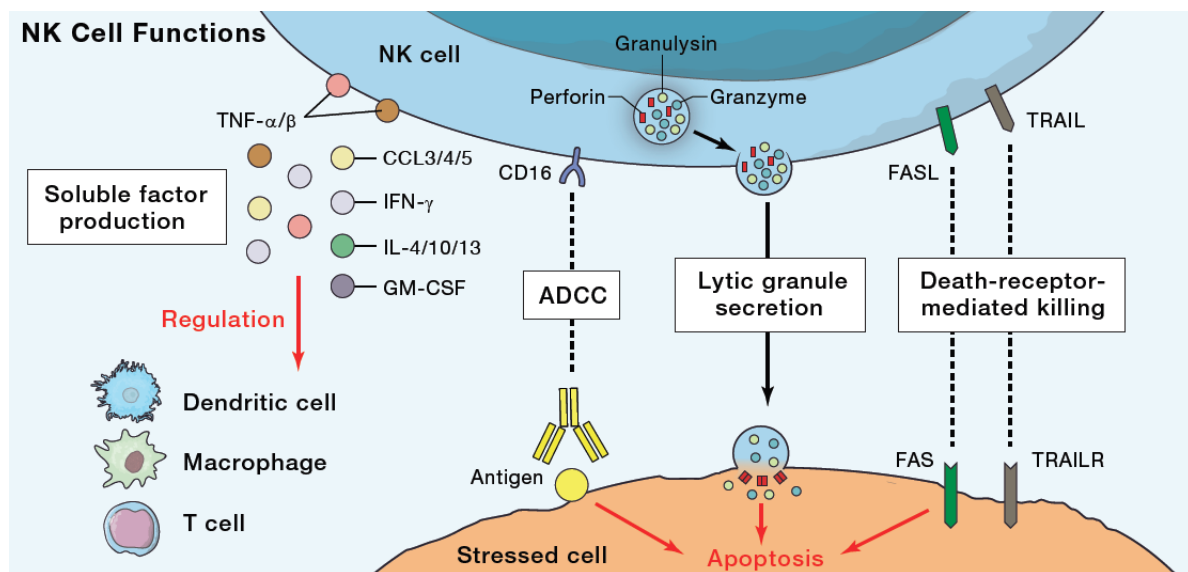


Figure 10. NK cell functions: cytotoxicity and cytokine secretion. NK cells are equipped with two weapons. Cytotoxicity is completed through three pathways: 1. production of lytic granules containing an arsenal of molecules including perforin, granzymes and granulysin in human (Clayberger and Krensky, 2003); 2. engagement of several TNF superfamily members, such as FASL and TRAIL which can induce apoptosis of target cells; 3. Antigen-dependent cellular cytotoxicity (ADCC) through Fc receptor CD16 by target cells coated with antibodies. Besides, NK cells can also secrete numerous cytokines especially IFN- γ , chemokines (CCL3, CCL4, CCL5) and GM-CSF shaping the response of immune responses by interacting with DCs, macrophages and T cells. Adapted from Crinier et al. (2020).

1.4.1 Killer's weapon: cytotoxicity

Mature NK cells are morphologically characterized as large granular lymphocytes similar to cytotoxic T lymphocytes (CTLs). Once the NK cell encounters a target cell, an immunological synapse will form and NK cell granules containing both perforin (a pore-forming protein) and granzymes (a family of proteolytic enzymes) will be released to mediate target cell death ([Voskoboinik et al., 2015](#)). Perforin can form pores in target cell membranes with an internal diameter of 16-22nm, which enable granzymes to diffuse across the plasma membrane into the cytosol of target cells and initiate apoptosis.

Perforin plays an essential role in immune surveillance of cancer as demonstrated by deletion of the gene encoding perforin in C57Bl/6 (B6) and BALB/c mice ([Smyth et al., 2000](#); [Street et al., 2002, 2004](#)). Granzymes, that are proapoptotic serine proteases, not only induce indiscriminate protein digestion but also activate caspase-driven cell death pathways ([Baran et al., 2009](#)). Among these, granzyme B is the most powerful pro-apoptosis granzyme with its robust capacity to cleave target cell proteins at sites after aspartate residues as target cells can be killed within few minutes by apoptosis when exposed to small amount of granzyme B and perforin ([Sutton et al., 2000](#)). Granzyme A cleaves proteins at sites after basic amino acids but in a slower rate than granzyme B. Other mechanisms of target-cell killing have been described, involving FASL (CD178) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, also known as TNFRSF10)-dependent receptors ([Smyth et al., 2002](#)). TRAIL has also been implicated in responses against cancer ([Finnberg et al., 2007](#); [Cretney et al., 2002](#)).

1.4.2 Killer's weapon: cytokine secretion

NK cells are potent producers of cytokines, especially Th1-type cytokines (including IFN- γ and TNF), in response to target cell interaction or to cytokine stimulation in the microenvironment. These cytokines are important for the control of infections by intracellular bacteria, and contribute to shaping the innate and adaptive immune

response. Apart from IFN- γ and TNF, NK cells can also secrete granulocyte macrophage-colony stimulating factor (GM-CSF), IL-10 and chemokines (e.g. CCL3, CCL4, CCL5). In human, CD56^{dim} and CD56^{bright} NK cells have usually been described as two distinct subsets, cytolytic and cytokine producing, respectively (Cooper et al., 2001). However, several observations claimed that there is no clear separation between the two populations regarding the functions. Upon target cell recognition, CD56^{dim} NK cells are highly cytotoxic but can produce cytokines as well (Fauriat et al., 2010b; De Maria et al., 2011). In contrast, CD56^{bright} NK cells can acquire cytotoxic activity and produce cytokines as well (Fauriat et al., 2010b; Takahashi et al., 2007).

Cytokines mainly function to recruit other immune cells, like DCs, macrophages and T cells, to the inflammation site and induce activation and proliferation of these cells (Vivier et al., 2011). IFN- γ , which is an early and potent factor produced by NK cells, has many effects on the immune response, including induction of MHC class II molecules on antigen presenting cells, activation of macrophage bactericidal activities and induction of T helper 1 (Th1) cells, as well as effects on angiogenesis. Macrophage activation by NK cell-derived IFN- γ has been shown to be essential for resistance to chemical carcinogenesis in a mouse model of primary tumorigenesis (Morvan and Lanier, 2016).

IFN- γ and perforin together provided optimal protection in 3 separate tumor model emphasizing the important role of IFN- γ (Street et al., 2001) in cancer development. Herein, both perforin and IFN- γ play critical and independent roles in protecting the host, but IFN- γ appears to play an early role in protection from metastasis (Street et al., 2001). These studies suggest that cytokines from NK cells mediate both innate and adaptive immune responses to control spontaneous tumors.

1.5 NK Cell Memory

NK cells have historically been considered as short-lived cytolytic cells that rapidly respond against pathogens and tumors and then undergo cell death. In recent years,

however, NK cells have been shown to develop immunological memory in response to previously encountered pathogens and in non-infectious settings (O'Sullivan et al., 2015). BrdU incorporation assay found that much lower number of memory NK cells incorporated BrdU than that of naive NK cells suggesting memory NK cells undergo basal turnover at a lower rate and a far greater lifespan than NK cells in naive mice (Sun et al., 2010).

During the acute phase of MCMV (mouse cytomegalovirus) infection in C57BL/6 mice, Ly49H receptors can specifically bind to the MCMV-encoded glycoprotein m157 expressed on infected target cells, driving the expansion of virus-specific NK cells (Arase et al., 2002). Moreover, when adoptively transferred into Ly49H deficient mice, these Ly49H⁺ NK cells undergo robust antigen-driven expansion against MCMV infection. This first "expansion" phase is followed by a second "contraction" phase where the vast majority of effector NK cells undergo apoptosis while the long-lived and self-renewing antigen-specific memory cells maintain and are ready for the third "memory" phase (Figure 11). The pool of memory NK cells can be recovered several months after infection in a variety of peripheral tissues (Sun et al., 2009a). In addition to the evidence showing the memory response takes place during MCMV infection, several studies supported that NK cells also participate in secondary response against other viruses, like herpes virus 2 (HSV-2 (Abdul-Careem et al., 2012) and influenza (van Helden et al., 2012).

Analogous to their T cell counterparts, three signals are also required for the antigen-specific NK cell proliferation during viral infection, that is: recognition of antigen by antigen-specific receptors (signal 1; e.g. Ly49H-m157), signaling through co-stimulatory receptors (signal 2; DNAM-1), and pro-inflammatory cytokine signaling (signal 3; IL-12, IL-18) (O'Sullivan et al., 2015). During the contraction phase, Bcl-2 and Bim were reported to co-deliver signals to repress NK cell survival thus limiting the size of the memory NK cell pool. Modulation of IL-15 signaling mediated by microRNA-155 also regulates the effector and memory NK cell maintenance. There is also evidence demonstrating that NK cell memory can be induced by cytokine stimulation alone during influenza infection (van Helden et al., 2012). NK cells pre-activated with pro-inflammatory cytokines (e.g. IL-12, IL-18, IL-15) display

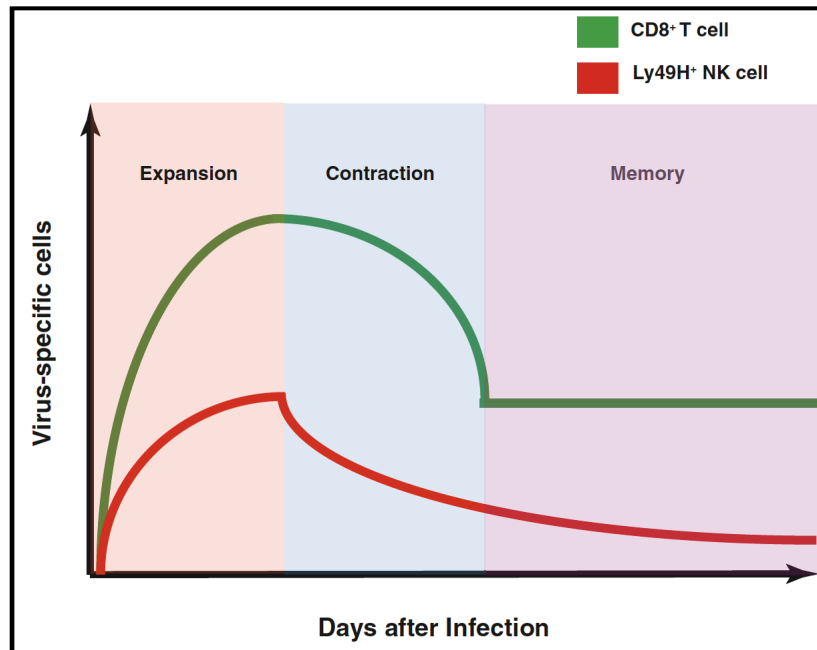


Figure 11. Three phases of NK and T cell upon virus infection. Upon exposure to cognate antigens, both CD8⁺ T cells and NK cells undergo three similar phases: expansion, contraction and memory. During the "expansion" phase, CD8⁺ T cells and Ly49⁺ NK cells clonally expand and differentiate into effector cells followed by a rapid "contraction" phase when the infection is under control and a majority of cells undergo apoptosis to form a small but stable pool of surviving cells that enter the third "memory" phase. Compared to CD8⁺ T cells, NK cells undergo a slower and sustained contraction phase before establishing a pool of memory NK cells. Adapted from O'Sullivan et al. (2015).

enhanced anti-tumor efficacy when adoptively transferred into mice. Interestingly, this memory-effect can persist in the host for up to 3 months suggesting that NK cells might provide long-lasting protection against tumors in therapeutic settings (Ni et al., 2012).

Human NK cells also display adaptive properties similar to what has been shown in mice. NK cells expressing the CD94-NKG2C activating receptor are present at a higher frequency in human cytomegalovirus (HCMV)-seropositive healthy individuals compared to the negative ones, and NKG2C positive NK cells increased in numbers in patients undergoing acute HCMV infection suggests a prolific expansion of this subset, a process resemblance to the clonal expansion of adaptive immune cells (Gumá et al., 2006; Lopez-Vergès et al., 2011). However, Blockade of CD94/NKG2C on NK cells or silencing of their ligand HLA-E in infected fibroblasts greatly impaired expansion of NKG2C⁺ NK cells, suggesting an essential role of CD94/NKG2C/HLA-E axis in the expansion of NKG2C⁺ NK cells in response to HCMV infection (Rölle

[et al., 2014](#)). Notably, a recent study found that adaptive NKG2C⁺ NK cells recognized many HCMV strains encoding variable UL40 peptides. These peptides could control the population expansion and differentiation of adaptive NKG2C⁺ NK cells in combination with pro-inflammatory signals ([Hammer et al., 2018](#)).

2 Transcription Factors Important for NK cell Development and Function

2.1 Generalities on Transcription Factors

The term transcription factor (TF) first referred to proteins involved in transcription and/or capable of altering gene-expression level. However, the term has been recently reserved for proteins capable of (1) binding DNA in a sequence-specific manner and (2) regulating transcription, guiding gene expression through recruiting other co-factors (Figure 12) (Lambert et al., 2018). Binding of a TF can change the shape of the DNA in a manner that either blocks or promotes the binding of another TF. For example, binding of CTCF (CCCTC-binding factor) to DNA is known to impact the 3D structure of chromatin thus affecting gene transcription (Fu et al., 2008; Kim et al., 2007). Other than binding to DNA loci directly, TFs can also impact transcription through the recruitment of either activating or repressive co-factors (Reiter et al., 2017).

A. DNA-binding domains



B. Effector domains

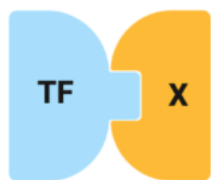


Figure 12. Schematic of a prototypical TF. TFs mainly function in two ways. (A) On one hand, they contain at least one domain that can bind to a specific DNA motif which can be shared among different TFs, for example, T-box family proteins recognize a similar T-box motif. (B) On another hand, TFs can work as interacting proteins which recruit co-factors further promoting specific phases of transcription.

The transcriptional regulatory system plays an essential role in controlling all

biological processes, ranging from cell cycle progression, maintenance of intracellular metabolic and physiological balance (Simon et al., 2001; Dynlacht, 1997), to cellular differentiation and development (Accili and Arden, 2004; Bain et al., 1994). Breakdown of transcription regulatory networks results in numerous diseases including cancer (Furney et al., 2006), autoimmune diseases (Hu et al., 2011) and developmental disorders (Boyadjiev and Jabs, 2000). Moreover, the involvement of TFs is a principal requirement for varied phenotypes and evolutionary adaptation (Bustamante et al., 2005; Levine and Tjian, 2003).

TFs can have 1,000-fold or even greater affinities for specific binding sequences compared to other random sequences (Damante et al., 1994; Geertz et al., 2012). The ability of TFs to bind specific DNA sequences is often taken as an indicator of their ability to regulate particular downstream transcription and alter the protein expression pattern. The short and related sequences that can specifically bind to a certain TF are termed as "motifs", which can be used to identify potential binding sites. Motifs are typically displayed as a sequence logo where each of the four bases at each position has a score (Schneider and Stephens, 1990), and multiplying these scores for each base of a sequence yields the predicted relative affinity of the TF to that sequence. The sequence preferences and binding sites of TFs can be assessed by a wide variety of techniques both *in vitro* and *in vivo* (Jolma and Taipale, 2011); Table 1 summarizes the most prevalent methods and their advantages and weaknesses. Mutation of TFs in the DNA-binding domain (DBD) can inhibit their binding to specific "motifs", interfering with gene expression and even resulting in deleterious diseases. It also explains why genomic loci-encoding TFs are enriched for ultra-conserved elements (Bejerano et al., 2004) and are depleted of common variation within their DBDs (Barrera et al., 2016), a backup employed for pivotal TFs to avoid potential damage.

Hematopoietic lineages are highly dependent on TFs largely shared between different populations. The development and effector functions of NK cells are governed by a series of TFs. E proteins, IRF-2, Ets1 and Nfil3 are among TFs known to drive the commitment to early stage of NK cells, while T-bet, Eomes, Runx3 are critical for the later differentiation and maturation. The proteins involved in NK cell development

	Method	Description	Features				
			Capability of de novo motif discovery (approx. length in base pairs with high information content)	Identifies genomic binding locations of a TF	Can measure effect of CpG Methylation	Can measure cooperative binding and/or multimers	
High-throughput	Protein Binding Microarray (PBM)	A GST-tagged TF is bound to a glass slide that has ~41,000 spots of short immobilized DNA sequences. Fluorescence-based detection of bound spots and k-mer enrichment analysis yields motifs.	✓ (< 12 bp)	✗	✓ Methyl-PBM	✓	
	Bacterial one-hybrid	TF binding sites are selected in bacterial cells from a randomized library that is cloned in front of selectable marker genes. Can be reversed to select proteins able to bind a constant DNA sequence using a library of variant protein sequences.	✓ (< 14 bp)	✗	✗	✗	
	SELEX-based methods	Systematic evolution of ligands through exponential enrichment (SELEX) involves adding TFs to a DNA pool containing many randomized sequences and selecting for binding in multiple rounds. Related methods include HT-SELEX, SELEX-seq, and Bind-n-Seq. Selection can be performed using affinity tags, or molecular trapping on a microfluidic platform (SMILE-seq).	✓ (< 25 bp)	✗	✓ Methyl-HT-SELEX	✓ CAP-SELEX ✓ SMILE-seq	
Mid-throughput	In Vitro Methods	DAP-seq	Single step SELEX using a library of fragmented genomic sequences. Sequence diversity is less than HT-SELEX, but genomic sequences that have co-evolved with the TF are included.	✓ Limited by skewed distribution of genomic sequences	✓ Peaks are not necessarily indicative of <i>in vivo</i> binding	✓ AmpDAP-seq	✗
		HITS-FLIP	Uses an Illumina sequencer's flowcell as a PBM chip to measure binding to orders of magnitude more DNA sequences.	✓ (< 17 bp)	✗	✗	✗
		Spec-seq	Single step SELEX using a synthesized library of degenerate sequences of interest. The lower complexity library is useful for quantitatively measuring effects of binding site mutations using sequencing.	✓ Limited by number of sequences assayed	✗	✓ Methyl-Spec-seq	✗
		MITOMI	A microfluidic device is used to isolate DNA-protein complexes from free DNA instantaneously to accurately measure the relative binding affinities of TFs to ~10,000 individual sites.	✗	✗	✗	✗
Low-throughput	In Vivo Methods	EMSA	Tests if a DNA sequence is bound by a protein by observing a shift in the electrophoretic migration of DNA.	✗	✗	✓	✓ EMSA-FRET
		DNA footprinting	DNA is incubated with a TF and then degraded using DNase-I, resulting in cuts in all positions except those that were protected by the bound TF.	✗ Useful for validating known binding sites	✗	✓	✓
		ITC, SPR, MSTP	Isothermal titration calorimetry (ITC), Surface plasmon resonance (SPR) and Microscale thermophoresis (MSTP) measure the binding affinity of TF-DNA interactions.	✗	✗	✓	✓
		ChIP-based assays	Proteins are crosslinked to DNA using formaldehyde and precipitated with an antibody. Bound DNA is detected with qPCR, microarray (ChIP-chip), or sequencing (ChIP-seq). The ChIP-Exo variant incorporates exonuclease treatment to enhance resolution.	✓ Limited by skewed distribution of genomic sequences, and inability to distinguish direct from indirect binding	✓	✓ ChIP + Bisulfite-sequencing	✓ Re-ChIP
		DamID-seq	A TF is expressed in mammalian cells as a fusion to bacterial Dam-methylase. The enzyme methylates a consensus sequence in close proximity to the TF's binding sites, which can be mapped using restriction enzymes and high-throughput sequencing.	✓	✓	✗	✓ Split DamID-seq

Table 1. Experimental methods for determining and validating TF-binding specificities. *In vivo* and *in vitro* methods currently used to experimentally derive and confirm TF-binding sites and motifs. **Adapted from Lambert et al. (2018).**

can also participate in regulating NK cell function, including T-bet, GATA3, Runx3, and E protein. Additional TFs including STAT1, STAT4, Zbtb32 and AhR play specific roles in effector functions of mature NK cells in the periphery (Sun, 2015; Hesslein and Lanier, 2011). The TFs that are involved in NK cell developmental stages and

functional responses are summarized in [Figure 13](#).

2.2 Ets-family: Ets-1, PU.1, and Mef

The Ets (E26 transformation specific) family of TFs is mainly characterized by the common DNA-binding domain (Ets-domain), and can be further sub-classified based on the DNA-binding domain ([Sharrocks, 2001](#)). Ets-family factors are universally found in a variety of cell types, and are involved in various biological processes including cellular activation, differentiation and oncogenesis. Ets-1 ([Barton et al., 1998](#)), PU.1 ([Colucci et al., 2001](#)) and Mef ([Lacorazza et al., 2002](#)) are three Ets-family factors that are implicated in NK cell development and functions.

Ets-1 is a proto-oncogene that is expressed in many cell types including lymphoid cells. Deletion of Ets-1 results in strong defects in NK cell development with very low numbers of total NK cells in the spleen, lymph nodes and almost undetectable NK cells in BM, indicating an essential role of Ets-1 in early NK cell development, especially in the transition from CLPs to NKP ([Barton et al., 1998](#); [Ramirez et al., 2012](#)). Ets-1 can promote the expression of critical TFs like T-bet and Id2, and NK activating receptors including Nkp46, Ly49H and Ly49D at the earliest stages of NK cell development ([Ramirez et al., 2012](#)). Further investigation of NK cell effector functions showed that Ets-1 deficient NK cells (CD3⁻DX5⁺) in the spleen were unable to kill the NK target cells YAC-1 or RMA-S, and exhibited reduced *in vivo* production of IFN- γ ([Barton et al., 1998](#)). Paradoxically, NK cells from *Ets-1*^{-/-} mice are hyper-responsive to cytokines and have increased expression of inhibitory Ly49 receptors including Ly49E and Ly49G2 ([Ramirez et al., 2012](#)). Thus, Ets-1 regulates a broad gene expression program in NK cells that relates to target cell recognition while abating cytokine-driven activation.

PU.1 (purine rich box-1) is a member of the Ets-family transcription factors widely expressed in immune cells, including myeloid lineages, B cells, and Th2 CD4⁺ T cells. Ets-1 and PU.1 share 35% similarity in DNA-binding region and Ets-1 is upregulated in PU.1 deficient NK cells, but PU.1 and Ets-1 seem to have

non-redundant roles ([Garrett-Sinha et al., 2001](#)). Given the fact that *PU.1*^{-/-} mice are embryonic lethal or immediately die after birth, PU.1 deficient fetal liver cells — a source of HSCs — were transferred into *Rag2/γc*^{-/-} mice in order to study the role of PU.1 in NK cells. These transferred HSCs from *PU.1*^{-/-} fetal liver could generate functional NK cells, but low numbers of NKPs and iNK cells compared to controls. *PU.1*^{-/-} NK cells also showed defective expression of Ly49 family (e.g. Ly49D and Ly49A) and failed to proliferate in response to IL-2 and IL-12, but were normal in terms of reactivity against YAC-1 cells ([Colucci et al., 2001](#)). Mechanistically, CD45-deficient NK cells phenocopy PU.1 deficient ones, and CD45 was proven to be a direct target of PU.1 ([Anderson et al., 2001](#)), suggesting that PU.1 could regulate NK cells through modulating CD45 expression. Thus, PU.1 is not absolutely required for NK cell development but regulates NK cell differentiation and homeostasis.

Mef, encoded by *Elf4* gene, is also expressed in multiple immune cells including both the myeloid and lymphoid lineages. Mef was shown to be involved in NK cell development by using *Mef*^{-/-} mice. Mef deficient mice had lower but still detectable NK cells in the periphery. *Mef*^{-/-} NK cells have defects in IFN-γ production and cytotoxicity upon stimulation with Poly I:C. However, Mef is not essential in CD8⁺ T cells for the ability to kill target cells. Mef can directly target the promoter region of the *Prf1* gene encoding perforin, which is essential to transactivate the promoter and thus promote perforin expression ([Lacorazza et al., 2002](#)).

2.3 Nfil3

Nfil3 (also named as E4bp4) contains a basic leucine zipper (bZIP) motif that binds DNA and is widely expressed by hematopoietic lineages including NK cells, NKT cells, macrophages and DCs ([Gascoyne et al., 2009](#)). Nfil3 is a critical regulator of the common helper-likelike innate lymphoid cell progenitor (CHILP) prior to ILC subset commitment. Accordingly, mice knockout for the *Nfil3* gene showed a severe block in ID2⁺ CHILP and PLZF⁺ ILC progenitors leading to impaired development of fetal and adult ILC subsets including both ILC1 and NK cells ([Xu et al., 2015](#)). The developmental defect in the BM occurs between NKP and iNK cell stages with E4bp4

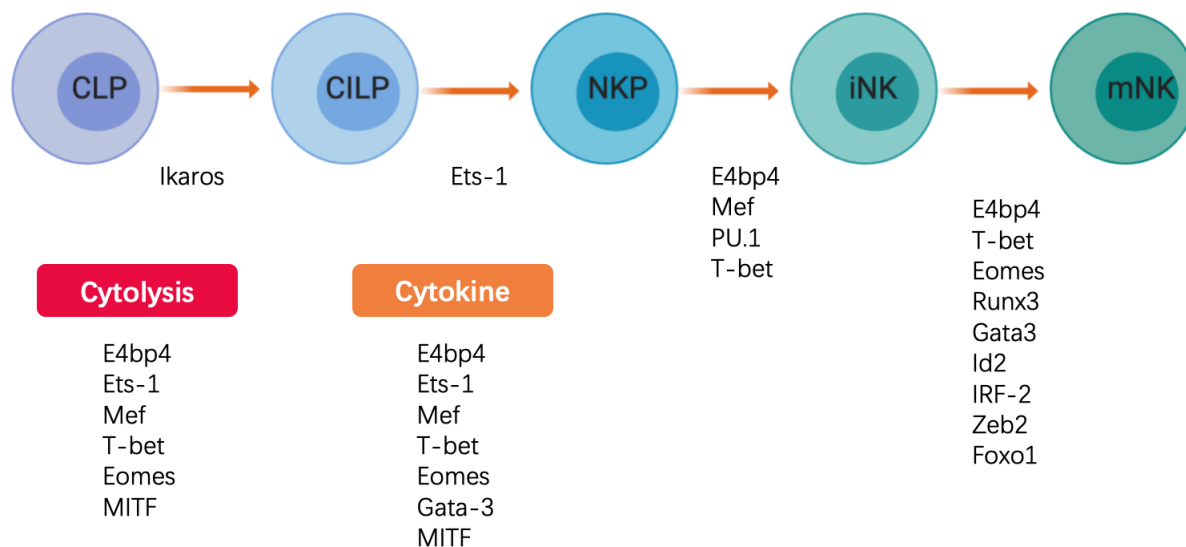


Figure 13. TFs in NK cells development and functions. On the upper part, TFs are listed under the transition stages in which they play a role. On the lower part, TFs are listed next to the functions divided into two parts: cytotoxicity and cytokine secretion. CLP, common lymphoid progenitor; CILP, common innate lymphoid progenitor; NKP, NK precursor; iNK, immature NK cell; mNK, mature NK cell. **Adapted from Hesslein and Lanier (2011).**

expression level gradually increasing after iNK stage. Almost no NK cells were detected in the periphery of *Nfil3* deficient-mice (Gascoyne et al., 2009; Kamizono et al., 2009).

Overexpression of *Nfil3* in BM progenitor cells rescued the defect brought by IL-15 receptor deficiency suggesting that *Nfil3* is a key factor downstream of IL-15 signaling through IL-15-PI3K-AKT-mTOR pathway (Yang et al., 2015a). In addition, retroviral overexpression of *Id2* or *Eomes* in *Nfil3*-deficient NK cells largely restored NK cell development suggesting that *E4bp4* might act via *Id2* or *Eomes* (Gascoyne et al., 2009; Male et al., 2014). However, the exact mechanism of the IL-15-*Nfil3*-*Id2*-*Eomes* axis remains unclear. Recently, Notch was also identified as a downstream target of *Nfil3* as the total lack of NK cell development from *Nfil3*^{-/-} progenitors was completely rescued by exposure to Notch peptide ligands (Kostrzewski et al., 2018). Regarding their function, the few NK cells detected in the spleen and BM of *Nfil3*^{-/-} mice were unable to kill YAC-1 targets or produce IFN- γ upon stimulation indicating the important role of *Nfil3* on NK cell cytotoxicity (Gascoyne et al., 2009; Kamizono et al., 2009).

2.4 Id2 and E-box proteins

Inhibitor of DNA-binding (Id) protein family consists of 4 members, Id1-Id4. These proteins contain an HLH dimerization domain but lack a basic DNA binding domain resulting in their incapacity to bind DNA themselves. Alternatively, they need to heterodimerize with other DNA-binding proteins, such as E-box proteins (E12, E47, HEB, and E2-2), to prevent them from binding to DNA (Benezra et al., 1990). Both Id2 and Id3 are highly expressed in lymphocytes including NK cells (Kee et al., 2000). In *Id2*^{-/-} mice, NK cells are dramatically decreased but NKp numbers are normal in the BM suggesting that Id2 participates in NK cell development after the NKP stage (Yokota et al., 1999; Boos et al., 2007). Interestingly, deletion of the TF Tox, which is upstream of Id2, results in a similar phenotype as *Id2*^{-/-} on NK cells. However, ectopic expression of Id2 was not able to restore NK cell development in the absence of Tox indicating the role of Tox in NK cell development is not solely dependent on Id2 (Aliahmad et al., 2010). Id2 partly functions through heterodimerizing with E2A protein, blocking its binding to DNA sites essential for T/B cell development thus favoring the NK cell development (Boos et al., 2007). Id2 expression is upregulated during NK cell maturation to tune E-protein activity. Additionally, Id2 is also required to regulate IL-15 receptor signaling and homeostasis of NK cells by repressing multiple E-protein target genes including *Socs3* (Delconte et al., 2016). However, E2A is not the only TF that binds to Id2 as deletion of E2A in *Id2*^{-/-} mice was not sufficient to restore the NK cell maturation in the periphery (Boos et al., 2007). Other E-protein like HEB might be involved as well. Moreover, NK cells from *Id2*^{-/-} mice are defective in producing IFN- γ in response to cytokine stimulation (Yokota et al., 1999).

2.5 T-box Family: T-bet and Eomes

2.5.1 The T-box family of transcription factors

T-box genes were first identified in 1927 (Dobeovolskaia-Zavadskaia, 1927) with the discovery of a mutation in the *Brachyury* (or *T*, for short-tail) gene, which caused embryonic lethality in homozygotes and truncated tail in heterozygotes (Papaioannou,

2001). A family of related proteins was then uncovered characterized by transcriptional activity and a conserved DNA-binding motif which was first defined as a common sequence having high affinity for Brachyury (Bollag et al., 1994). Based on the T-box binding domain that spans 180-200 amino acid residues and binds specific loci, the T-box family can be divided into five subfamilies: the T, Tbx1, Tbx2, Tbx6 and Tbr1 (Figure 14). T-box proteins are reportedly expressed in a highly specific manner

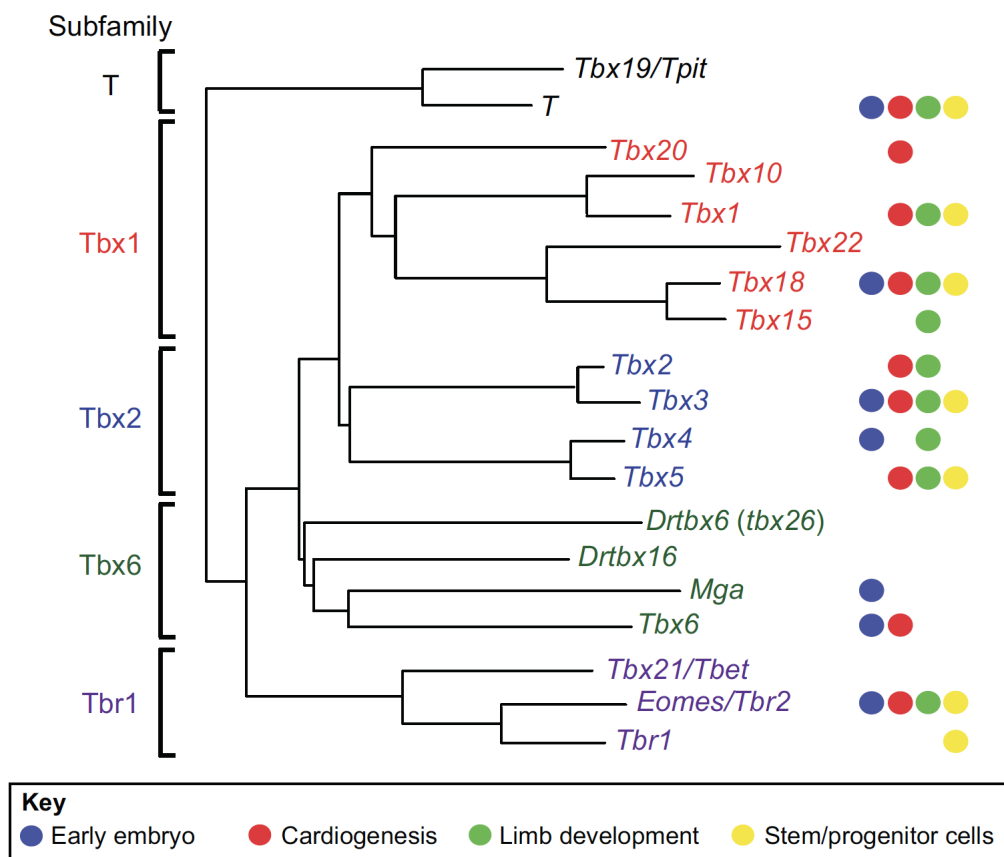


Figure 14. Phylogenetic tree of the T-box gene family in vertebrates. Colored circles on the right indicate involvement of particular genes in different areas including early embryo, cardiogenesis, limb development and stem/progenitor cell development. All of the genes are represented in human and mouse with the exception of *Danio rerio* (*Dr*) *tbx16*, which is present in birds and frogs (*VegT*) but not mammals, and *Drtbx6*. The T-box family is composed of five subfamilies: T, Tbx1, Tbx2, Tbx6 and Tbr1. Adapted from Papaioannou (2014).

and exhibit widespread involvement throughout development in vertebrates, including brain development (Tbr1, Eomes), craniofacial development (Tbx1, Tbx10, Tbx15, Tbx22), thymus (Tbx1), liver (Tbx3), Lung (Tbx2, Tbx4, Tbx5), mammary gland (Tbx2, Tbx3) and immune system (Tbx21, Eomes). In addition, increasing evidence show that they also participate in certain cancers. Interestingly, the function of T-box genes is supposed to be dose-dependent as heterozygotes display a milder

phenotype compared to the corresponding homozygotes (Papaioannou, 2014).

T-bet, encoded by the gene *Tbx21*, was first described in the immune system in 2000 and is exclusively expressed in cells of hematopoietic origin. It was defined as a key regulator of the Th1 lineage commitment (Szabo et al., 2000). T-bet promotes IFN- γ production by directly binding to the promoter of the *Ifng* gene (Townsend et al., 2004), and orchestrates Th1 cell differentiation while preventing that of other T helper cells (e.g. Th2, Th17) through interaction with other key factors (Villarino et al., 2010; Lazarevic et al., 2011; Zhu et al., 2012). Diminished IFN- γ secretion is also observed in T-bet-deficient CD8⁺ T cells (Sullivan et al., 2003). However, the expression of T-bet is not restricted to T cells unlike its name "T-box expressed in T cells" suggests. Indeed, DCs also require T-bet expression to prime the antigen-specific CD4⁺ T cells (Wang et al., 2006). In the absence of T-bet, B cells also show defects in class switching to immunoglobulin G2a/c (IgG2a/c), and T-bet is also a key TF associated with "age-associated B cells" and development of autoimmunity (Myles et al., 2017). T-bet deficiency negatively affects the terminal maturation and homeostasis of NK and iNKT cells (Townsend et al., 2004). Collectively, T-bet has broad actions in multiple cell types including innate and adaptive lymphocytes and also participates in mediating resistance to infection (Kallies and Good-Jacobson, 2017; Pritchard et al., 2019).

Eomesodermin (**Eomes**), another T-box transcription factor, is encoded by the *Tbr2* gene and shares great similarities (e.g. structure, expression pattern and functions) with T-bet. Eomes was originally recognized as a key factor for mesoderm formation during vertebrate embryogenesis (Ryan et al., 1996). Homozygous deletion of Eomes results in embryonic lethality in mouse (Russ et al., 2000). Reiner and colleagues showed that ectopic expression of Eomes could compensate the deficiency of T-bet expression and restore functions of effector CD8⁺ T cells, including cytotoxicity and production of IFN- γ , perforin and granzyme B (Pearce et al., 2003). Further study of mice with compound mutations of T-bet and Eomes (*Tbx21*^{-/-} *Eomes*^{+/-}) suggested that Eomes could cooperate with T-bet to induce the expression of CD122, the β chain of IL-15 receptor, which is indispensable for development of CTLs (cytotoxic T lymphocytes) and mature NK cells (Intlekofer et al.,

T-bet and Eomes are absent in common ILC precursors (Klose et al., 2014; Hoyler et al., 2012; Lim et al., 2017), and they are weakly expressed in NKP cells in the BM regulated by several TFs (e.g. Nfil3 for Eomes, Runx3 and Ets-1 for T-bet) (Levanon et al., 2014; Male et al., 2014; Ramirez et al., 2012). T-bet expression is kept at a low level in the BM compartment compared to the periphery and, interestingly, the expression of T-bet rapidly decreases when mature NK cells recirculate from the periphery to the BM. By contrast, Eomes is already expressed at a high level in BM NK cells (Daussy et al., 2014). *T-bet*^{-/-} NK cells express a higher level of Eomes whereas ectopic expression of T-bet results in lower Eomes level (Daussy et al., 2014). It was also found an aberrant expression pattern of T-bet and Eomes in the absence of Tsc (a repressor of mTOR), as in *Tsc*^{-/-} mice T-bet was up-regulated while Eomes and CD122 level decreased (Yang et al., 2016). In human, the levels of T-bet and Eomes also correlate with advancing stages of human NK cell maturation, with Eomes expression decreasing and T-bet expression increasing as NK cells acquire their functional maturity (Luetke-Eversloh et al., 2014; Collins et al., 2017).

The role of T-bet in NK cells was first demonstrated using T-bet deficient mice (*T-bet*^{-/-}) which have slightly higher NK cells numbers in the BM but reduced numbers in the spleen, liver and peripheral blood compared to the WT mice (Townsend et al., 2004). In T-bet deficient mice, NK cell proliferation is increased but their survival is compromised. *T-bet*^{-/-} mice show a more immature phenotype dominated by the CD27⁺CD11b⁻ population, suggesting a role of T-bet in the later maturation rather than early stages of development (Townsend et al., 2004). Interestingly, the mTORC2-Akt-Foxo1 axis has been shown to repress the expression of T-bet to regulate NK cell maturation and functions as well (Deng et al., 2015; Yang et al., 2018).

The study of *Vav1*^{Cre/+} X *Eomes*^{fl/fl} mice that are deficient for Eomes in the hematopoietic lineage showed a major role of NK cell development in spleen and blood and less importantly in liver, lymph node and bone marrow. Moreover, NK cells were completely absent in *T-bet*^{-/-} *Vav1*^{Cre/+} *Eomes*^{fl/fl} mice that lack both Eomes and T-bet, suggesting distinct and complementary role of T-bet and Eomes in NK cell development (Gordon et al., 2012). Deregulated expression of CD11b and other maturation markers like Ly49 receptor in Eomes-deficient mice suggests that Eomes

is also involved in NK cell maturation but in a different manner compared to T-bet (Gordon et al., 2012).

Function

ChIP (chromatin immunoprecipitation) assays in the YT NK cell line showed that IFN- γ was a direct T-bet target (Beima et al., 2006). IFN- γ production also positively correlates with T-bet and Eomes protein levels (Gill et al., 2012), but either deficiency of T-bet or Eomes does not affect IFN- γ secretion in NK cells (Way and Wilson, 2004; Gordon et al., 2012) which is quite different from what is observed in T cells (Pearce et al., 2003). Thus, T-bet and Eomes may compensate each other in NK cells. Another possible explanation is that other TFs may compensate the effect of T-bet and Eomes to induce or maintain IFN- γ secretion upon stimulation with IL-12 and IL-18. T-bet has a redundant role in NK cell cytotoxic activity, however, Eomes seems to be indispensable in regulating several NK cell inhibitory receptors such as Ly49R (Gordon et al., 2012). Eomes can also promote the secretion of GzmB and Prf1 by facilitating histone acetylation at the gene locus (Intlekofer et al., 2005; Araki et al., 2008). Moreover, T-bet and Eomes are both necessary for the expansion of virus-specific NK cells during mouse CMV infection through an inducible deletion system (Madera et al., 2018). T-bet can be upregulated by IL-12 through STAT4 binding to enhancer region of *Tbx21* gene (Madera et al., 2018).

As discussed above, S1PR5 is an essential player in NK cell trafficking from BM and LN to the periphery. It was reported that T-bet could directly bind to the 3' of the gene *S1pr5* thus regulating NK cell migration by modulating the expression of S1PR5 (Jenne et al., 2009). CXCR3, involved in NK cell entry into inflamed LNs, is another potential T-bet target gene as suggested by early study in a human NK cell line (Beima et al., 2006). Whether Eomes behaves the same way in regulating migration as T-bet in NK cell needs further investigation.

2.6 Zeb family transcription factors: Zeb1, Zeb2

The zinc-finger E homeobox-binding (Zeb) family of transcription factors consists of two members: Zeb1 and Zeb2. Zeb proteins are composed of several domains including two zinc-finger domains at both ends of the protein (NZF and CZF), a centrally located homeodomain (HD), a SMAD-binding domain (SBD) and a repressor CtBP (C-terminal binding protein) interactive domain (CID) (Figure 16) (Vandewalle et al., 2009; Scott and Omilusik, 2019). Two zinc-finger domains act as DNA-binding regions and have high affinity to the consensus motif E-box, CANNTG. Zeb proteins mainly function as transcriptional repressors and are involved in driving epithelial to mesenchymal transition (EMT) through repression of genes essential for epithelial cell-cell junction (Vandewalle et al., 2005), a process required for embryogenesis and tumor metastasis (De Craene and Berx, 2013; Brabletz and Brabletz, 2010; Goossens et al., 2011). Both *Zeb1*- and *Zeb2*-knockout mice are embryonic lethal emphasizing the importance of Zeb proteins in development. Zeb proteins have universal roles across immune lineages including DCs, macrophages, monocytes, B, T and NK cells (reviewed in (Scott and Omilusik, 2019)).

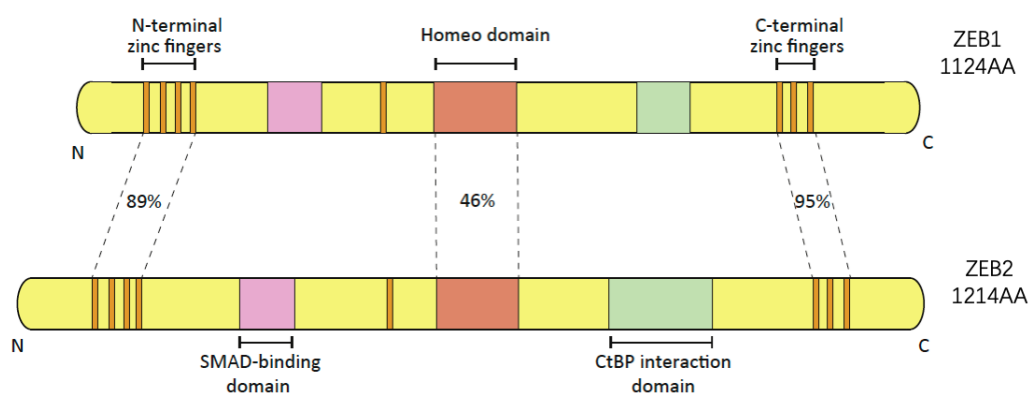


Figure 16. The ZEB family of transcription factors. Schematic representation of the two ZEB family members ZEB1 and ZEB2 in human. ZEB1 consists of 1124 amino acids (AA) whereas ZEB2 has 1214. These two proteins are highly homologous and both are characterized by two zinc-finger domains allocated at each side of the proteins. In addition, they share a Homeo domain, a SMAD-binding domain and a C-terminal binding protein (CtBP) interaction domain. The percentages indicated in each domain denote the extent of amino acid homology. Adapted from Scott and Omilusik (2019).

Zeb1 has been previously involved in NK cell development through a forward genetic screen (Arnold et al., 2012), but the underlying mechanisms require further

investigation. Zeb2 is highly expressed in NK cells (Figure 17) and its expression gradually increases upon maturation in both human and mouse NK cells (van Helden et al., 2015). Zeb2-deficient NK cells are very similar in phenotype to *T-bet*^{-/-} NK cells, in terms of impaired NK cell maturation, survival, and migration from the bone marrow. Furthermore, transgenic expression of Zeb2 in *T-bet*^{-/-} mice partially restored a normal NK maturation pattern suggesting that Zeb2 is an essential transcriptional effector downstream of T-bet (van Helden et al., 2015). In the absence of Zeb2, mice were more susceptible to melanoma outgrowth with decreased numbers of NK cells especially mature NK cells in the lung (van Helden et al., 2015). However, the responsiveness to chemokines, cytotoxicity potential and cytokine secretion remained normal or even increased in Zeb2-deficient NK cells (van Helden et al., 2015). T-bet also plays a role in inhibiting cancer metastasis by regulating the longevity and function of NK cells (Werneck et al., 2008). Moreover, Zeb2 is essential for the generation of both memory CD8⁺ T cells and memory NK cells during MCMV infection (Lau et al., 2018).

2.7 Runx proteins: Runx3

The Runx proteins contains a conserved Runt motif essential for DNA-binding and can heterodimerize with the protein CBFβ facilitating the binding efficiency. The Runx family consists of three highly homologous proteins in mammalian cells: Runx1, Runx2 and Runx3 (Ito et al., 2015). Both Runx1 and Runx3 are expressed in NK cells, while Runx2 is expressed at very low levels in the hematopoietic system. Runx3 is a dominant factor in NK cells as it is abundantly expressed in both immature and mature NK cells (Ohno et al., 2008; Otto et al., 2003). Conditional knockout of Runx proteins in NK cells revealed an important role of these TF in NK cell maturation with a decreased expression of the maturation marker CD11b but normal NK cell numbers. However, the NK cell functions of cytokine secretion and cytotoxicity remained unchanged or elevated (Ohno et al., 2008). Similarly, in *Runx3*^{-/-} mice, there was no difference in total NK cell number compared to normal mice, but the NK cells were more immature with higher number of NK cells found at immature stage (CD27⁺CD11b⁻) (Levanon et al., 2014). The modest impact of Runx3 knockout on

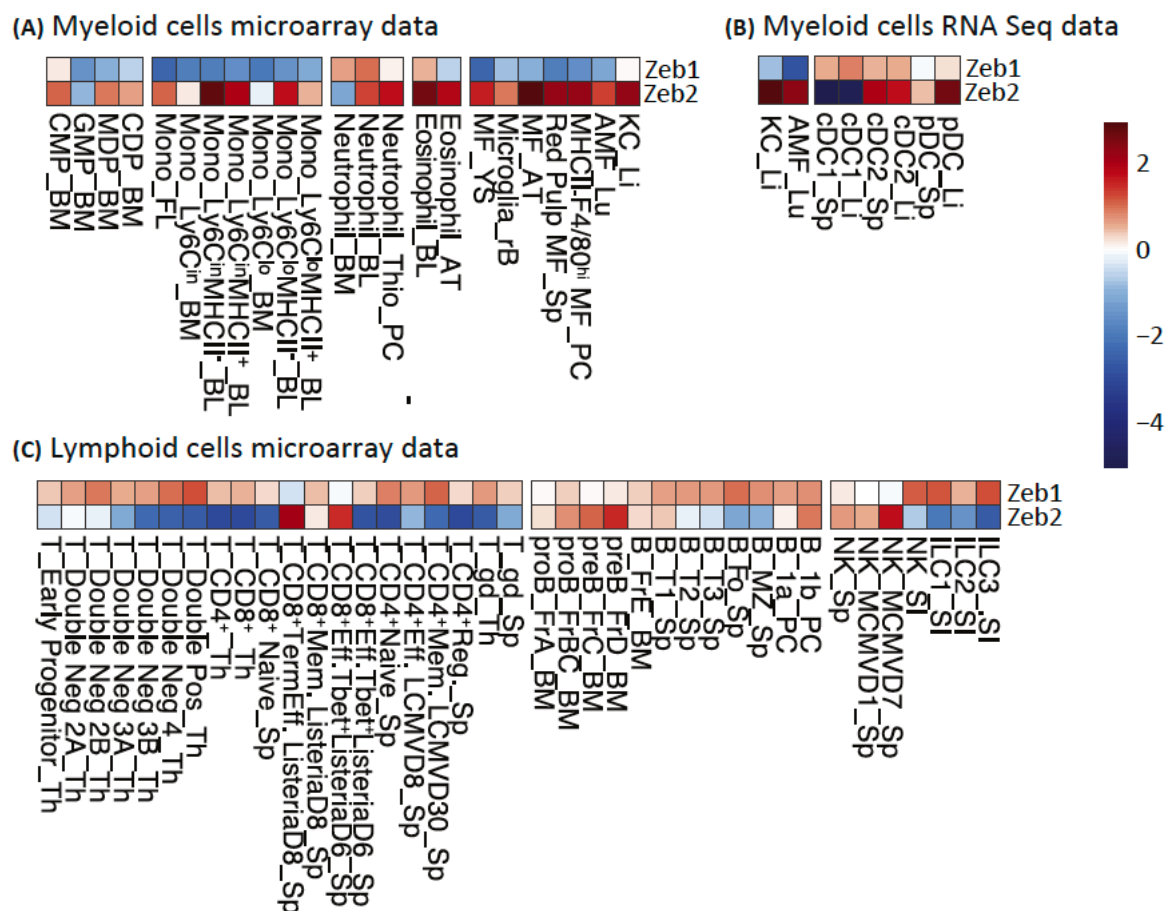


Figure 17. Zeb1/2 expression in murine immune system. Three heatmaps show the relative transcript levels of Zeb1 and Zeb2 across mouse immune cells including both myeloid cells and lymphoid cells. Data are collected and compiled from microarray and bulk RNA-seq data. Zeb1 and Zeb2 do not display the same profile regarding the expression level. In general, Zeb2 is highly expressed across myeloid cells. On the contrary, Zeb1 has higher expression in lymphoid lineages than myeloid ones. **Adapted from Scott and Omilusik (2019)**

NK cell development could be due to partial redundancy with Runx1. Further investigation demonstrated that Runx3 was also essential for normal development of ILC1 cells by controlling their survival especially in the intestine as a dramatic decrease in ILC1 numbers was observed in Runx3-deficient mice (Ebihara et al., 2015).

Chromatin immunoprecipitation of Runx3 in mouse NK cells showed that CD122 is a direct target of Runx3 in the promoter region, which explains the fact that Runx3-deficient NK cells had decreased proliferative response to IL-15 stimulation (Ohno et al., 2008). 90% of peaks found in the Runx3 ChIP-seq have a Runx binding motif suggesting that Runx3 directly bind to target genes rather than through indirect

effects (Levanon et al., 2014). Enrichment of T-box and Ets motif in Runx3 binding sequences and differential gene expression analysis of wild-type versus Runx3-deficient NK cells suggests that Runx3 can recruit both TFs during NK cell activation (Levanon et al., 2014). Recently, Eomes was discovered to interact with Runx3 to promote T cell memory formation through epigenetic modulation (Istaces et al., 2019). Except for CD122, Runx3 also binds to the promoter region of other NK-related genes, including Ly49 receptors and NKp46 (Ohno et al., 2008; Lai and Mager, 2012).

2.8 Gata proteins: Gata3

Gata family TFs are zinc finger DNA binding proteins that are broadly necessary for the regulation of proliferation, cell survival and differentiation of diverse tissues. The Gata family can be divided into two categories: hematopoietic GATA factors (i.e. Gata1, Gata2 and Gata3) and endodermal Gata factors (i.e. Gata4, Gata5, Gata6) (Zheng and Blobel, 2010).

Gata3 is the most studied Gata factor in NK cells. Gata3 was originally described to participate in the regulation of thymic T cell development and Th2 differentiation (reviewed in Tindemans et al. (2014)). *Gata3*^{-/-} mice are embryonic lethal; using fetal liver chimera mice, Gata3 was found to be essential for both NK cell development and functions. In the absence of Gata3, NK cells in the bone marrow have an incomplete repertoire of Ly49 receptors and lower expression of CD11b. Although Gata3-deficient mice display normal number of NK cells in the spleen, most of the cells found there are immature. *Gata3*^{-/-} NK cells have defects in IFN- γ production during pathogen infection (e.g. *Listeria monocytogenes*), and nevertheless retained normal cytotoxicity when co-cultured with YAC-1 target cells (Samson et al., 2003). T-bet that is important for IFN- γ production and Hlx, a downstream target for T-bet, were reduced in Gata3 deficient NK cells although the relationship between Gata3 and T-bet still remains unclear (Samson et al., 2003; Mullen et al., 2002). Gata3 is also involved in migration from bone marrow to liver as less NK cells were found in the liver in the absence of Gata3 (Samson et al., 2003), of

which the phenotype could be revisited in light of the complexity of liver ILC populations, as now established. Gata3 expression is particularly enriched in NK cells derived from thymus, and the lack of Gata3 protein impairs thymic NK cell development in mice (Vosshenrich et al., 2006), although thymic NK cells may now be classified as ILC1s because they express CD127 which is normally present in ILC1s rather than NK cells (Vosshenrich et al., 2006).

2.9 Blimp1

Blimp1, encoded by the gene *Prdm1*, is a repressive transcription factor that plays a crucial role in the differentiation of plasma B cells and CD8⁺ effector T cells (Crotty et al., 2010). It is expressed by NK cells throughout their development and is required for NK cell maturation and homeostasis by controlling proliferation and granzyme B production (Kallies et al., 2011). Cytokine stimulation could induce the expression of Blimp1 in NK cells including IL-15 and pro-inflammatory cytokines IL-12 or IL-21 (Kallies et al., 2011). Unlike T cell or B cells, Blimp1 is not regulated by Bcl6 or IRF4 in NK cells, but is reduced by loss of T-bet. Another TF, Zbtb32 can antagonize Blimp1 thus permitting NK cells to proliferate in response to viral infection (Beaulieu et al., 2014). Mutations in Blimp1 have been found in many NK cell-associated cancers indicating that Blimp-1 may be an important regulator for development of NK cell leukemia and lymphoma (Karube et al., 2011). In addition, Blimp1 was identified to cooperate with Hobit to instruct tissue retention in diverse tissue-resident lymphocytes including liver-resident NK cells (Mackay et al., 2016; Kallies et al., 2011).

2.10 Other TFs

Apart from the essential factors discussed above, there are a number of other TFs involved in NK cell development and functions as well, but for which few details are known regarding the mechanism of actions e.g. **Ikaros**, **IRF-2**, **MITF**. Ikaros-deficient mice lack several lymphoid lineages including NK cells, T cells and B cells (Wang

[et al., 1996](#)). Loss of functional NK precursors in *Ikaros*^{-/-} mice indicates the role of Ikaros in early NK cell differentiation ([Boggs et al., 1998](#)). Aiolos, another member of Ikaros family, is identified as a regulator of NK cell maturation that could cooperate with T-bet or Blimp1 ([Holmes et al., 2014](#)). Bone marrow and peripheral NK cells were strongly reduced in *IRF-2*^{-/-} mice with almost normal proliferation but accelerated apoptosis that was distinct from the early IL-15-dependent expansion, while the capacity of cytotoxicity was normal compared to the wild-type cells although the IFN- γ was mildly blocked ([Taki et al., 2005](#)). MITF (microphthalmia transcriptional factor) is essential for NK cell-mediated cytotoxicity, IFN- γ production and response to cytokine stimulation ([Kataoka et al., 2005](#)). However, the mutation in the binding domain of MITF did not perturb the NK cell development in terms of numbers ([Ito et al., 2001](#)).

3 NK-like Immune Cells or NK Paralogues

3.1 CD8⁺ Cytotoxic T cells

NK cells and CD8⁺ cells are both generated via CLP precursors (Kondo et al., 1997) and require cytokine signals especially through γ -chain receptors for survival and homeostasis. CD8⁺ cells undergo positive and negative selection through interaction with TCR and peptide-MHC class I complex expressed on thymic epithelial cells or DCs (Adachi et al., 1998). Similarly, NK cell are selected or educated through engagement of various MHC class I ligands, a process called "missing-self" recognition (Kiessling et al., 1975). However, if T cells do not go through TCR selection they will die by neglect while NK cells do not die and are exported to the periphery as anergic cells (Johansson et al., 2005). Over the past years, the role of many TFs such as Runx factors and Notch has been described in T cell development (Collins et al., 2009; Maillard et al., 2005). CD4⁺ and CD8⁺ T cell development are driven by GATA4, THPOK and RUNX1, RUNX4, respectively (Collins et al., 2009; Bosselut, 2004).

In terms of function, NK cells mirror the functions of CD8⁺ cytotoxic T cells. During infection, CD8⁺ cells can produce potent pro-inflammatory cytokines IFN- γ and perforin, granzymes similar to NK cells, which ensures the host to effectively defend against pathogens. Together with NK cells, CD8⁺ T cells can directly target infected cells during virus infection (Sun et al., 2011). Specific sub-population of NK cells will undergo dramatic proliferation upon reactivation with the same stimulus, for example Ly49H⁺ NK cells during MCMV infection (Sun et al., 2009a; Robbins et al., 2004). Similarly, a subset of memory T cells that expressed NKRs was also found in both mice and humans against tumor (Ugolini et al., 2001; Anfossi et al., 2001). Thus, both CD8⁺ cytotoxic T cells and NK cells harbor the capacity of immune memory and longevity (Sun et al., 2010). However, the longevity is not clear in NK cells as NK cells treated by IL-15 in macaques are short-lived and do not persist for long term (Lugli et al., 2010).

3.2 Innate Lymphoid Cells (ILCs)

The prototypical ILC (innate lymphoid cell) populations are NK cells and lymphoid tissue-inducer (LTi) cells ([Mebius et al., 1997](#)) discovered in 1975 and 1997 respectively. LTi cells can induce the development of secondary lymphoid organs through instructing mesenchymal stromal cells to produce factors essential for hematopoietic cell migration to occupy developing lymphoid organs. Recently, several ILC populations i.e. ILC1, ILC2, ILC3 have been identified which are developmentally related to NK cells and LTi cells. They also depend on common cytokine receptor γ -chain (γ_c) and Id2 ([Seillet et al., 2016](#); [Xu et al., 2019](#)). These ILC subsets rely on signals through IL-7 receptor subunit- α (IL-7R α) for their development and maintenance.

ILCs are categorized into three groups based on the cytokine secretion pattern and distinct TF involvement ([Spits et al., 2013](#); [Serafini et al., 2015](#)) with ILC1, ILC2, ILC3 mirroring CD4⁺ T helper (Th)1, Th2 and Th17 respectively ([Koyasu and Moro, 2012](#)). Group 1 ILCs comprise NK cells and ILC1 cells as they both require T-bet for development and produce great amount of IFN- γ upon activation. Group 2 contains only one subset, ILC2s that respond to large extracellular parasites and allergens ([Moro et al., 2010](#); [Hoyler et al., 2012](#); [Mjösberg et al., 2012](#)). Group 3 ILC cells include NCR⁻ ILC3s, NCR⁺ ILC3s, and LTi cells which can produce IL-17 and/or IL-22. In 2018, a new nomenclature for ILCs was proposed to classify ILCs into five subsets — NK cells, ILC1s, ILC2s, ILC3s and LTi cells — based on their development, effector functions and phenotypes that mirror adaptive CD8⁺ and CD4⁺ T cells ([Vivier et al., 2018](#)). Although both conventional NK cells and ILC1s express T-bet and are capable of producing IFN- γ and TNF, NK cells also express Eomes and Eomes-dependent perforin and granzymes ([Figure 18](#)).

3.2.1 ILC1 cells

Development

CILPs are derived from CLPs and can give rise to both NK cells and ILCs ([Yang et al.,](#)





Stimuli		Mediators	Immune function
Tumors, intracellular microbes (Virus, bacteria, parasites)	→ 	IFN- γ Granzymes Perforin	Type 1 immunity (Macrophage activation, cytotoxicity)
Large extracellular parasites and allergens	→ 	IL-4 IL-5 IL-13 IL-9 AREG	Type 2 immunity (Alternative macrophage activation)
Mesenchymal organizer cells (Retinoic acid, CXCL13, RANK-L)	→ 	RANK Lymphotoxin TNF IL-17 IL-22	Formation of secondary lymphoid structures
Extracellular microbes (Bacteria, fungi)	→ 	IL-22 IL-17 GM-CSF Lymphotoxin	Type 3 immunity (Phagocytosis, antimicrobial peptides)

Figure 18. Immune functions of ILCs. The best-known stimuli, mediators and immune functions of each ILC subset are shown. NK cells and ILC1s react to intracellular pathogens and tumors through similar mediators including IFN- γ , granzymes and perforin. ILC2s respond to large extracellular parasites and allergens via Th2-type cytokines (e.g. IL4, IL-5, IIL-13). ILC3s combat extracellular microbes like bacteria and fungi armed with IL-22 and IL-17. LTI cells are involved in the formation of secondary lymphoid structures. **Adapted from Vivier et al. (2018).**

2015b). Id2 is essential for both CHILP and NKP development, which acts as an inhibitory factor reducing the activity of E-box TFs (E2A, E2-2, and HEB) thus blocking the potential of early progenitors to develop into T or B cells. An Id2⁺ CHILP can differentiate into ILCP (innate Lymphoid cells precursor), which can further develop into all ILC subsets, but not NK cells (Klose et al., 2014) (Figure 19). However, what are the key factors that make the final decision to develop into CHILP or NKP is still unknown. Other than Id2, Nfil3 and Gata3 have been shown to be critical at the ILCP stage (Seillet et al., 2014; Yagi et al., 2014). Gata3 can repress B cell fate by blocking EBF1 and facilitate the differentiation of T and ILC cells, however, Nfil3, like Id2, plays a broader effect in driving ILCs differentiation (García-Ojeda et al., 2013).

In mice, ILC1s are first detected before birth, whereas NK cells emerge two to three weeks after birth (Diefenbach et al., 2014; Daussy et al., 2014). Early stages of ILC1

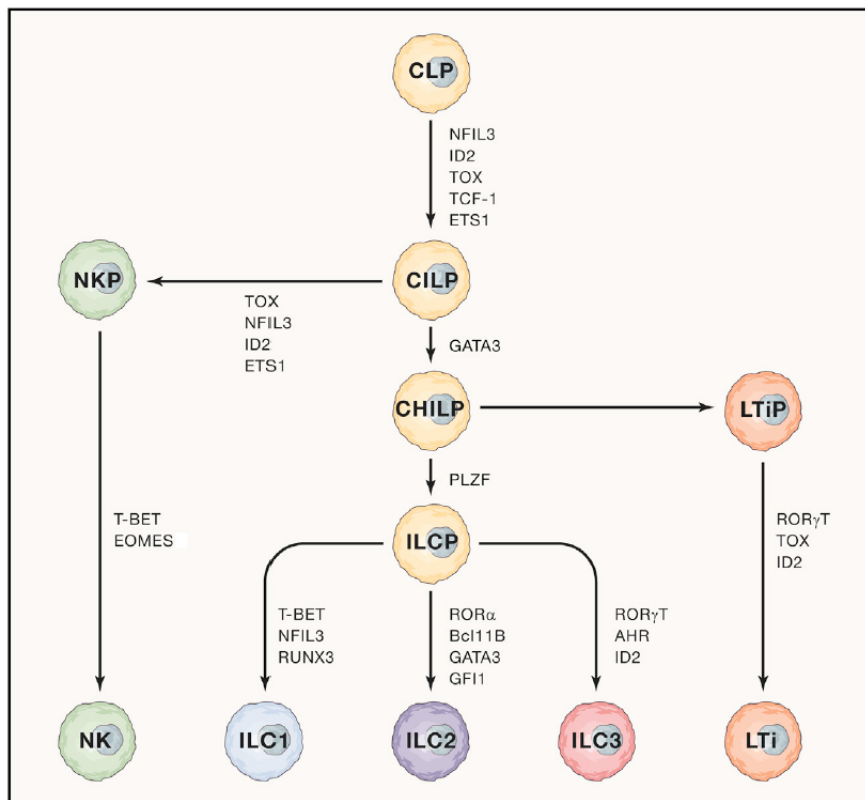


Figure 19. Development of ILCs. Schematic overview of innate immune cell development. The five sub-populations of ILCs are originally derived from CLP which give rise to all lymphoid lineages. With the regulation of TFs, CLPs differentiate into CILPs and further branch into two pathways either NKP cells or CHILPs that give rise to LTIps (lymphoid tissue inducer progenitors) and ILCP (innate lymphoid cell precursors). LTIps can differentiate into LTI and ILCPs into ILC1, ILC2 or ILC3 dependent on the different transcriptional programs. **Adapted from Vivier et al. (2018).**

lineage development requires the transcription factor PLZF as evidence shows that they arise from CHILPs that express PLZF although ILC1 cells themselves do not express PLZF (Constantinides et al., 2015). By contrast, fate-mapping reporter mice showed that NK cells could be derived from both PLZF⁻ and PLZF⁺ progenitors (Constantinides et al., 2015). Interestingly, intestinal CD127⁺ ILC1s are dependent on IL-15 instead of IL-7 which is essential for other ILCs, suggesting that ILC1s are more similar to NK cells (Klose et al., 2014). In mice, ILC1 cells are highly dependent on T-bet, whereas NK cells are still present in T-bet-deficient mice suggesting that NK cells are not strictly dependent on T-bet for their development (Townsend et al., 2004; Daussy et al., 2014). Eomes is expressed in NK cells and is essential for NK cell maturation as discussed above but is not necessary for ILC1 cells (Daussy et al., 2014; Gordon et al., 2012).

NK cells and ILC1 cells have different developmental pathways and functions. A comprehensive analysis of gene expression profiles of mouse ILCs and NK cells has been reported and PCA plot from RNA-seq showed that ILC1s are much closer to NK cells than to ILC2s or ILC3s, and the transcriptional profiles of NK cells and ILC1s are largely overlapping (Robinette et al., 2015). ILC1 cells preferentially express CD49a and TRAIL in both humans and mice, but the specificity of these markers is often lost upon stimulation and varies a lot under different tissue environment (Eberl et al., 2015; Vivier et al., 2018). Initial studies demonstrated that there existed two types of NK cells on top of TRAIL expression in the liver (Takeda et al., 2001), however, they were later categorized into two distinct subsets (Daussy et al., 2014). At steady state, CD49a⁺CD49b⁻Eomes⁻ ILC1s and Eomes⁺ NK cells are definitely two different sub-populations as ILC1s cannot develop into Eomes⁺ NK cells by adoptive transfer of Eomes⁻ ILC1s into the recipient mice (Daussy et al., 2014). However, CD49a expression can be induced on conventional NK cells during virus (Bezman et al., 2012) and parasite infection (Park et al., 2019), and in the tumor microenvironment (Cortez et al., 2017), which makes the discrimination between cNK cells and ILC1 cells complicated. For example, ILC1s in the liver selectively express TRAIL and VLA1 but there are no distinctive surface markers expressed in ILC1s from the spleen or small intestine, although the expression of Cxcr6 on ILC1s and of the Ly49R and KIRs on NK cells can be partially informative (Robinette et al., 2015).

Function

NK cells express high levels of perforin and are highly cytotoxic, whereas ILC1 cells are weakly cytotoxic in general and function as the first line of defense against infections by producing IFN- γ (Spits et al., 2016), as shown in the case of *T. gondii* (Klose et al., 2014) and *C. difficile* (Abt et al., 2015). Thus, NK cells may be termed as "cytotoxic ILC1s". In the context of *T.gondii* and *C. difficile* infection, T-bet-dependent ILC1s are critically involved although the role of NK cells in the control of infection cannot be excluded (Abt et al., 2015; Klose et al., 2014). Moreover, ROR γ ⁺ ILC3s-derived T-bet⁺ ILC1 cells can also provide protection against *Salmonella enterica* (Klose et al., 2013). In the context of *Helocobacter hepaticus*-induced colitis, ILC1s reportedly exerted an important role either protective or detrimental (Buonocore et al., 2010; Powell et al.,

2012).

NK cells are widely recognized to be essential for antitumor responses, however, whether ILC1s are also critical in cancer remains elusive as NK cells and ILC1s are difficult to discriminate from each other. NKp46⁺ ILC3s have a protective role against B16 melanoma cells in an IL-12-dependent manner and ILC3-to-ILC1 conversion requires IL-12. Thus, it is possible that ILC1s mediate immune response against tumor development (Eisenring et al., 2010). The conversion of NK cells into ILC1-like cells was also described during *Toxoplasma gondii* infection, although the ILC1-like cells are distinct from both steady-state NK cells and ILC1s in uninfected mice (Park et al., 2019). With respect to the heterogeneity of ILC1 population in different tissues and plasticity of ILC populations, the role of ILC1 cells in infection and cancer should be revisited.

3.2.2 ILC2 cells

ILC2 cells are defined by their capacity to produce Th2-associated cytokines including IL-4, IL-5, IL-13 in response to stimulation with IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (Moro et al., 2010; Neill et al., 2010). Unlike ILC1s and ILC3s that are tissue-resident cells, ILC2s are recently reported to circulate upon inflammation or infection probably with an alteration in their sensitivity to S1P-mediated chemotaxis, leading to lymphatic entry, blood circulation, and accumulation in many non-gut sites (Huang et al., 2017; Lombardi et al., 2016). ST2, a component of IL-33 receptor, is a conventional marker for tissue-resident ILC2s in mice, but in some tissues like the skin ILC2s do not express ST2, indicating the varied phenotype of ILC2s (Bal et al., 2016). The development of ILC2s sequentially requires Id2, IL-7 and the cytokine receptor common γ chain in early progenitors (Yokota et al., 1999; Cao et al., 1995; Moro et al., 2010), while the differentiation requires transcription factor GATA3, ROR α , TCF1, and Notch signaling as well (Furusawa et al., 2013; Hoyler et al., 2012; Wong et al., 2012; Mielke et al., 2013; Gentek et al., 2013). Gata3 has a general role in all helper-like ILC subsets development of multiple stages. Deletion of Gata3 at a later time point in mice results

in the impairment of ILC2 differentiation (Hoyler et al., 2012), while overexpression of Gata3 rescues ILC2 number (Wolterink et al., 2013). Gata3 is also required for human ILC2 cell development (Mjösberg et al., 2012). ROR α is highly expressed in ILC2s and is required for ILC2 cell differentiation (Moro et al., 2010; Wong et al., 2012), but ROR α mutation does not abrogate the cytokine production (e.g. IL-5, IL-13) in response to IL-13 (Furusawa et al., 2013). Apart from BM, ILC2 cells can also differentiate from committed T cell precursors in the thymus. This pathway is suppressed by E-proteins including E2A and Heb (Qian et al., 2019). However, thymus-derived lung ILC2s of E-protein-deficient mice showed different transcriptomes, proliferative properties and cytokine responses from wild-type counterparts (Qian et al., 2019). Other factors like vitamin A and its metabolites derived from food control the balance of ILC2s and ILC3s (Spencer et al., 2014).

Beyond IL-4, IL-5 and IL-13, ILC2s also produce IL-6 and IL-9 and are important in host resistance against parasitic worm infection (Neill et al., 2010; Price et al., 2010; Monticelli et al., 2011) and viral infections (Monticelli et al., 2011). ILC2s accumulate in the lung after acute infection with influenza virus to repair damaged tissue by secreting epidermal growth factor family member amphiregulin (Monticelli et al., 2011). However, ILC2s participate in lung immunopathology during allergic asthma (Chang et al., 2011). Furthermore, in collaboration with eosinophil-derived cytokines, ILC2s can promote the differentiation of adipocytes progenitors into white fat or beige fat thereby affecting thermal homeostasis (Lee et al., 2015). TFs like Bcl11b and Gfi1 regulate the responsiveness of ILC2 cells to "alarmins" like IL-22 within inflamed tissues and enhance type 2 cytokine secretion (Califano et al., 2015; Spooner et al., 2013).

3.2.3 ILC3 cells

ILC3s are defined by their dependency on the transcription factor ROR γ t and their robust production of IL-22 and IL-17A that play important roles in innate immune response against extracellular bacterial and fungal infection (Diefenbach, 2013; Vonarbourg et al., 2010). Indeed, ROR γ t acts as a dominant TF in ILC3s, by

regulating multiple downstream genes encoding the signature cytokines like IL-17A and IL-22 and also aryl hydrocarbon receptor (AHR) that is required for the survival and function of ILC3 cells (Qiu and Zhou, 2013). *Id2* is not only important for selection of ILCP lineage as indicated before, but is also required for ILC3 cell development in the gut after ROR γ t expression (Satoh-Takayama et al., 2010). Substantial reduction of IL-22-producing-ILC3s found in *Rorc*^{Cre} X *Id2*^{fl/fl} mice resulted in higher susceptibility to *Citrobacte rodentium* compared to wild-type littermates (Guo et al., 2015). The generation of both ILC3s and ILC1s requires Runx3 that can directly bind to *Rorc* and affect the optimal expression of ROR γ t (Ebihara et al., 2015). Beyond ILC2s, Gata3 also regulates the development of ILC3s as Gata3-deficient chimeric mice lack all intestinal ROR γ t⁺ ILC3 cells and show impaired survival and defective capacity to produce IL-22 during early infection (Serafini et al., 2014). In addition, Gata3 positively regulates the balance between the transcription factors T-bet and ROR γ t thus serving as a critical factor in the development of NKp46⁺ ILC3 cells (Zhong et al., 2016)

ILC3s are abundantly found at mucosal sites, and IL-22 predominately secreted by ILC3s maintains intestinal homeostasis and promotes the proliferation of intestinal stem cells (Rankin et al., 2016; Aparicio-Domingo et al., 2015). IL-22 produced by ILC3s could induce epithelial cells to express anti-bacterial peptides such as Reg3 γ and Reg3 β and antiviral proteins thus protecting tissues from damage (Hernández et al., 2015). IL-22 is important to sustain the progression of colon cancer and colorectal cancer (Huber et al., 2012; Hernandez et al., 2018; Kirchberger et al., 2013). A sub-population of ILC3s found in the intestine express cell surface markers similar to NK cells, like NKp46, NKG2D, NKp44, or CD56. Fate-mapping studies have shown that NKp46⁺ ILC3s developed independently of NK cells and were differentiated from NKp46⁻ ILC3s (Crellin et al., 2010; Satoh-Takayama et al., 2010; Sawa et al., 2010). NKp46⁻ ILC3s can also produce the cytokine GM-CSF and are involved in colitis development (Pearson et al., 2016). In mice, NKp46⁺ ILC3 cells can produce IFN- γ like ILC1s and are dependent of T-bet for generation, however, human ILC3s do not produce IFN- γ (Sciumé et al., 2012; Klose et al., 2013).

3.2.4 LTi cells

LTi cells are very similar to ILC3 cells in terms of transcriptional regulation and cytokine production, particularly the dependency on ROR γ t for development and capacity to produce IL-22 and IL-17A following stimulation. They were first identified as innate lymphocytes in LN and Peyer's patch of E13.5-E15.5 mice (Mebius et al., 1997; Adachi et al., 1998), and they are crucial for the formation of secondary organs during embryogenesis. These cells do not express surface markers of NK cells (e.g. Nkp46) but express c-Kit and CCR6 instead. And LTi cells and ILC3 cells are supposed to differentiate along different developmental pathways (Figure 19).

3.3 iNKT

Natural killer T (NKT) cells are unconventional T cells that bridge innate and adaptive immune responses. NKT cells were first described as CD3⁺ T cells co-expressing NK1.1, a classic marker used to identify mouse NK cells. A more precise definition has been proposed for NKT cell afterwards: a subset of T cells that expresses a semi-invariant T cell receptor (TCR) (Lantz and Bendelac, 1994; Dellabona et al., 1994) and specifically recognizes lipid antigens presented by CD1d molecule, a non-polymorphic MHC class I-like antigen-presenting molecule (Godfrey et al., 2004, 2015). CD1d-restricted NKT cells can be further subdivided into two broad categories: type I NKT cells, referred to as "invariant NKT cells" or "iNKT cells", which express an invariant TCR α chain (TCR α -chain variable region 11 (TRAV11) and joining region 18 (TRAJ18) in mouse, and TRAV10 and TRAV18 in human), and a limited repertoire of TCR β chains (V β 8.2, V β 7, or V β 2 in mouse, V β 11 in human) (Lantz and Bendelac, 1994; Rossjohn et al., 2012); Type II NKT cells also recognize lipids presented by CD1d but express more diverse TCR chains compared to the type I NKT. Type II NKT cells are also named as "diverse NKT cells" (Rhost et al., 2012). iNKT cells can be sensitively detected using α -Galcer-loaded CD1d tetramers (Kawano et al., 1997) and numerous studies have used this reagents to study them. By contrast, the lack of sensitive reagents to identify type II NKT cells hampers the research on this cell type. In this introduction I will only focus on type I invariant NKT

cells.

3.3.1 Development of iNKT cells

iNKT cells are found in both lymphoid and non-lymphoid organs, including thymus, spleen, liver, BM, lung, adipose tissue and intestine (Figure 20) (Crosby and Kronenberg, 2018). iNKT cells are abundant in the liver accounting for up to 30-40% of the lymphocytes, while less than 1% in other tissues (Hammond et al., 2001). However, in human iNKT cells only represent 0.1% of T cells in PBMC and the numbers are shown to be of great variety between individuals (Chan et al., 2013). iNKT cells are tissue resident cells that contribute to both protective effects against pathogens and tissue homeostasis (Crosby and Kronenberg, 2018).

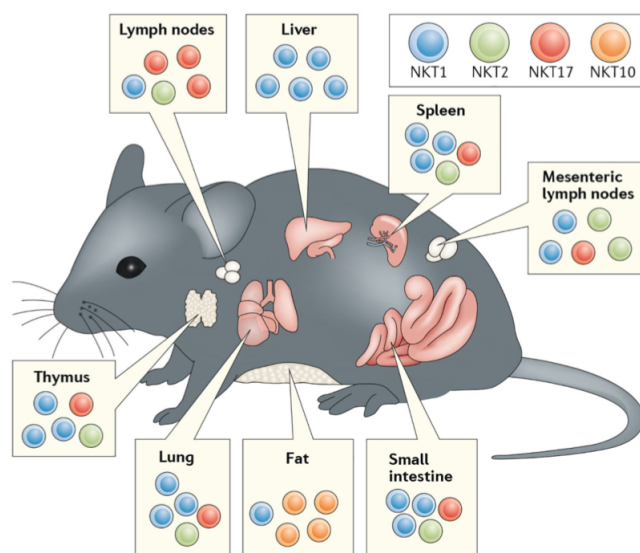


Figure 20. Tissue distribution of iNKT cell subsets in mice. Schematic overview of the distribution of iNKT subsets in lymphoid or non-lymphoid organs in C57BL/6 mice. NKT1, NKT2, NKT17, NKT 10 cells are indicated in blue, green, red and orange, respectively. The figure depicts the relative frequencies of each iNKT subsets in different tissue sites, including liver, lungs, intestine, and adipose tissue. NKT10 cells are exclusively found in adipose tissue (Wingender et al., 2015). Adapted from Crosby and Kronenberg (2018).

Mouse iNKT cells originally derive from CD4⁺CD8⁺ (DP) cortical thymocytes that branch away from the conventional T cell lineage in response to different TCR display and signal strength. iNKT cells undergo positive and negative selection similarly as CD4 or CD8 SP T cells. However, they also experience agonist selection in the thymus, which requires the engagement of agonist ligands stimulating higher TCR

signals (Stritesky et al., 2012). iNKT cell development mainly requires two types of signals from surface receptors, that is, TCR and SLAM family (Nunez-Cruz et al., 2008). Engagement of SLAM family recruits SAP and the Src family tyrosine kinase Fyn thus promoting signals critical for iNKT lineage commitment (Das et al., 2010).

Once positively selected, iNKT cells go through several developmental intermediates defined by marker CD24 (the heat stable antigen, HSA), CD44 and NK1.1 (Figure 21). Transcription factor PLZF expression is induced after selection of NKT

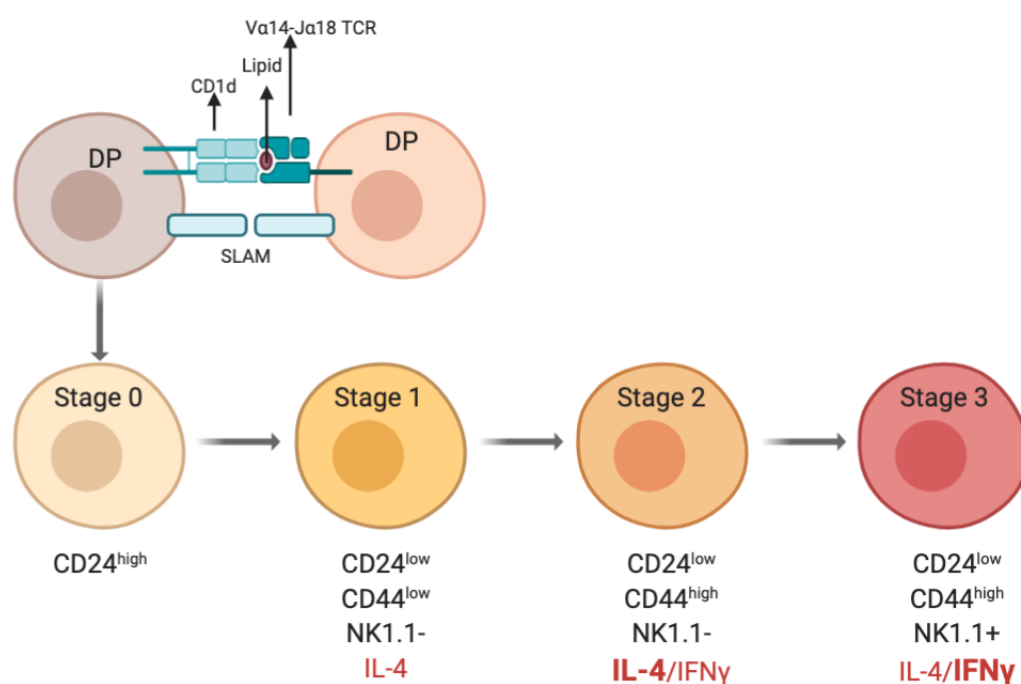


Figure 21. Differentiation of iNKT cell development. The well-recognized model for iNKT cell development requires two critical signals from double positive cells: recognition of TCR and lipid-CD1d complex and engagement of SLAM family that is essential for cell survival. Once positively selected, they are differentiated into iNKT cells undergoing four stages. Stage 0-3 iNKT cells can be discriminated by surface markers CD24, CD44 and NK1.1. And the specific cytokines produced by each subsets are denoted besides. Both stage 2 and stage 3 iNKT cells can produce IL-4 and IFN- γ , but iNKT cells from stage 2 mainly secrete IL-4 but stage 3 cells principally produce IFN- γ .

precursors (Savage et al., 2008) which is then controlled by another TF, Egr2 (Seiler et al., 2012). CD24^{high} iNKT cells do not undergo proliferation and are considered as stage 0, followed by three sequential stages that lose expression of CD24. Stage 1 cells are defined as CD24⁻CD44⁻NK1.1⁻ and proliferate robustly (Benlagha et al., 2002; Pellicci et al., 2002). The acquisition of the activation marker CD44 signifies the progress to stage 2, namely, CD44⁺NK1.1⁻. Finally, upon acquisition of NK1.1, iNKT

cells become fully mature and are recognized as stage 3 that stop proliferating and rapidly express other NK lineage markers such as NKG2D, Ly49A, Ly49C/I and Ly49G2 (Benlagha et al., 2002; Pellicci et al., 2002). NK1.1 expression is acquired either in the thymus or in the periphery, and stage 3 iNKT cells produce Th1-type cytokines controlled by T-bet and IL-15 (Townsend et al., 2004; Matsuda et al., 2006). During the transition from stage 0 to stage 1, a proportion of iNKT cells downregulate CD4, giving rise to a subset of NKT cells that are CD4⁻CD8⁻. However, CD8⁺ iNKT cells also exist in human and are highly cytolytic (Takahashi et al., 2002)

An alternative subdivision of NKT cells also exists. Indeed, iNKT cells can be distinguished as iNKT1, iNKT2, iNKT17 subsets (Figure 22) based on the expression pattern of TFs such as T-bet, Gata3, PLZF and RORγt (Lee et al., 2013). iNKT1 cells produce IFN-γ and are defined as T-bet⁺PLZF^{low}. iNKT2 cells produce IL-4 and are defined as GATA3^{high}PLZF^{high}RORγt⁻ cells. iNKT17 cells mainly produce IL-17 and are defined as RORγt⁺PLZF^{int} cells (Lee et al., 2013).

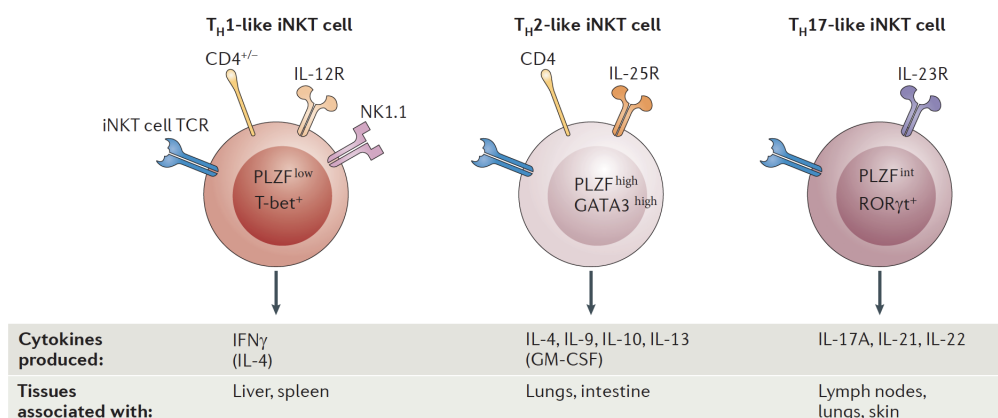


Figure 22. iNKT cell subsets. The figure compares three proposed major subsets of invariant natural killer T (iNKT) cells. Crucial transcription factors and surface markers associated with each subset are shown. These subsets are associated with the constitutive expression of cytokine receptors for interleukin-12 (IL-12), IL-23 and/or IL-25. The major cytokines produced by each subset and the tissues in which they are enriched are indicated. **Adapted from Brennan et al. (2013).**

3.3.2 Function of iNKT cells

Upon antigen encounter and TCR engagement or cytokine signaling, iNKT cells rapidly become effector cells and produce a broad range of cytokines including IL-12, IFN-γ,

IL-4, and IL-13 within minutes to hours (Crowe et al., 2003; Wilson et al., 2003). The pattern of cytokine secretion varies between different developmental stages. Stage 1 NKT cells produce IL-4 while NKT cells in stage 2 produce both IL-4 and IFN- γ , and mature NKT cells from stage 3 are capable to secrete more IFN- γ than IL-4 (Gadue and Stein, 2002). Besides, cytotoxicity and chemokine production are dominant in stage 3 NKT cells as well (Matsuda et al., 2006).

iNKT cells are supposed to act as adjuvant cells (Cerundolo et al., 2009) that potently activate DCs through CD40 ligand and cytokines, thus enhancing antigen presentation of DCs to MHC-restricted T cells (Fujii et al., 2004), promoting B cell response (Galli et al., 2003) and NK cell activation as well (Eberl and MacDonald, 2000). As CD1d is non-polymorphic between individuals, adoptive transfer of iNKT cells does not cause graft-versus-host disease. Therefore, a series of studies have been launched to investigate the therapeutic role of stimulated iNKT against CD1d⁺ tumor targets *in vivo* (Heczey et al., 2014; Wolf et al., 2018; Wingender et al., 2010).

3.3.3 Transcriptional regulation of iNKT cell development and function

PLZF

PLZF is encoded by the gene *Zbtb16* and is highly expressed by iNKT cells especially in the immature stage 0/1 population (Kovalovsky et al., 2008; Savage et al., 2008). Mice deficient for PLZF exhibit approximately 90% reduction in the iNKT cells numbers in the thymus, but have normal development of conventional $\alpha\beta$ T cells. The defect in iNKT cells could be due to a proliferative defect and an increased apoptosis (Kovalovsky et al., 2008; Savage et al., 2008). Cytokine production of iNKT cells was also impaired in *Plzf*^{-/-} mice (Kovalovsky et al., 2008; Savage et al., 2008). However, the mechanism of PLZF in regulating iNKT cell development and functions remains unclear.

Egr2

The Egr family of transcription factors consists of three members Egr1, Egr2, and Egr3 that are known targets of NFAT (nuclear factor of activated T-cells). TCR

engagement induces numerous signaling events, including release of calcium from intracellular stores and activation of the phosphatase calcineurin, which results in the dephosphorylation and subsequent nuclear translocation of NFAT family member and the subsequent regulation of NFAT target genes (Macian, 2005). In fact, deletion of *Cnb1*, the gene encoding the regulatory component of calcineurin, resulted in a dramatic reduction in thymic and peripheral iNKT cell numbers (Lazarevic et al., 2009). Further analysis on *Egr1*^{-/-}, *Egr2*^{-/-}, *Egr3*^{-/-} mice showed that the absence of *Egr2*, but not *Egr1* or *Egr3*, led to significant reductions in iNKT cells, but did not affect development of CD4⁺ or CD8⁺ cells. Moreover, *Egr2*^{-/-} mice had normal numbers and percentages of CD24⁺ cells (Stage 0) but had a block at the transition from Stage 0 to Stage 1 of iNKT cells (Lazarevic et al., 2009). Collectively, the calcineurin-NFAT-*Egr2* pathway is important in the development of the iNKT lymphocyte lineage.

Notch

Notch signaling, originally characterized to be major in cell-to-cell interactions during development (Radtke et al., 2013), is important for T cell lineage commitment and early thymic development, up to the double-negative 3 stage and also affects T cell effector functions as well as Treg expansion (Amsen et al., 2004; Kared et al., 2006; Maekawa et al., 2008). Thus, Notch regulates the development and functions of various T cell subsets *in vivo*. Analysis of the importance of Notch signaling in iNKT cells showed that Notch 1 and Notch 2 affected the development of iNKT cells in the thymus as well. Ablation of Notch 1 and Notch 2 (*N1N2*^{-/-}) in mice resulted in lower cytokine production by iNKT cells and increased apoptosis (Oh et al., 2015). iNKT cell populations are affected in *N1N2*^{-/-} mice with increased numbers of stage 2 and 3 iNKT cells, increased proportion of iNKT17 and altered expression of PLZF in stage1 iNKT cells (Oh et al., 2015).

c-Myb

The proto-oncogene *c-Myb* encodes a nuclear DNA-binding protein that is expressed primarily in hematopoietic tissues and acts as an activator and repressor of transcription. Deletion of *Myb* in the germline is embryonic lethal and thymocytes

lacking c-Myb have a profound developmental defect at the CD4⁻CD8⁻ DN stages (Allen et al., 1999). Abrogation of c-Myb expression at distinct stages of T-cell development reveals additional roles for c-Myb activity in early T cell development, including transition through the double-negative 3 stage, DP survival and differentiation into CD4 SP cells (Bender et al., 2004). Conditional knockout of c-Myb at the DP stage revealed an additional role for c-Myb in the generation of iNKT cells that was associated with impaired TCR α gene rearrangements (Hu et al., 2010). Additionally, c-Myb is essential for the expression of SLAM, Ly108, and SAP at the DP stage, these proteins being required for iNKT cell lineage commitment (Hu et al., 2010).

E protein and Id

The E protein family of basic-helix-loop-helix TFs includes HEB, E12, E47 (E2A), and E2-2, which bind as homo- or hetero-dimers to DNA at E-box sites and control the expression of genes essential for lineage development. Deletion of HEB or E2A specifically in DP thymocytes results in a major block in iNKT cell development at the CD24^{hi}CD44⁻NK1.1⁻ stage; iNKT cells fail to progress through stage 2 and 3. However, these deletions have minimal effects on the development of CD4⁺ or CD8⁺ T cells (D'Cruz et al., 2010; D'Cruz et al., 2014). E2A and HEB were shown to directly bind the promoter of PLZF and control its expression in stage 1 iNKT cells (D'Cruz et al., 2014). Furthermore, their negative regulators, the Id proteins (Id2 and Id3) also regulate iNKT cell development. A loss of Id3 led to an increased number of iNKT2, while both Id2 and Id3 were required for the formation of iNKT1 cells (Hu et al., 2013; Verykokakis et al., 2013).

T-box family

T-bet also plays an indispensable role in the final maturation stages of iNKT cells (Matsuda et al., 2006; Townsend et al., 2004). In the absence of T-bet, iNKT cell numbers are reduced and are blocked at stage 2. As thymic *T-bet*^{-/-} iNKT cells do not express CD122, a component of the IL-15 receptor, iNKT cells fail to proliferate in response to IL-15. In addition, T-bet is also required for effector functions of iNKT, such as cytokine production as IFN- γ is supposed to be a direct target of T-bet

(Matsuda et al., 2006; Townsend et al., 2004). In addition, Eomes is expressed at higher level in stage 0 than the other iNKT cell stages, and has recently been identified to regulate NKT cell ontogeny particularly the iNKT1 cell differentiation (Shimizu et al., 2019). However, mature iNKT cells do not express Eomes.

3.4 NK1.1⁺ $\gamma\delta$ T cells

$\gamma\delta$ T cells are the first T cells to appear in the thymus during fetal thymic ontogeny. In human peripheral blood, there are approximately 4% of $\gamma\delta$ T cells with a wide range between 0.5% and 16% (Groh et al., 1989). In adult mouse, 1-4% of total T cells are found to be $\gamma\delta$ T cells in the thymus and the secondary lymphoid organs. Besides lymphoid organs, $\gamma\delta$ T cells are present in non-lymphoid tissues including skin (O'Brien and Born, 2015), lung (Sim et al., 1994), intestine (Takagaki et al., 1989), liver (Gerber et al., 1999) and reproductive tract (Itohara et al., 1990). Several distinct subsets of $\gamma\delta$ T cells are observed in mouse and human, and they notably have different TCR uses and different patterns of tissue homing (Godfrey et al., 2015).

$\alpha\beta$ and $\gamma\delta$ T cells both originate from the CD4⁻CD8⁻ (DN) T cell. DN cells consist of 4 stages defined by c-kit, CD24 and CD44. Early T-cell-lineage progenitor (ETP) and DN2 cells proliferate robustly and acquire their first T cell characteristics (Allman et al., 2003). As the cells develop into DN3 T cells, they start to initiate TCR gene rearrangement and generate in-frame TCR gene rearrangements (Taghon et al., 2006). Successful TCR β selection endows the following expression of CD4 and CD8 to become DP cells, which then undergo positive and negative selection to generate CD4⁺ or CD8⁺ TCR $\alpha\beta$ T cells and NKT cells as well. Alternatively, DN3 T cells that succeed in making TCR γ and TCR δ chains directly develop into $\gamma\delta$ T cells without expression of CD4 or CD8 co-receptors (Rothenberg et al., 2008). It is widely recognized that the T cell fate largely depends on the strength of signals delivered by TCR, namely, DN3 cells receiving stronger TCR signal favoring $\gamma\delta$ T cells development and vice versa (Haks et al., 2005; Hayes et al., 2005). However, a recent study demonstrated that a specific subset CD5⁻NK1.1⁺ $\gamma\delta$ T cells could develop directly from DN2 stage in a Bcl11b-independent manner and was involved in

early defense against bacterial infection in the periphery ([Hatano et al., 2017](#)).

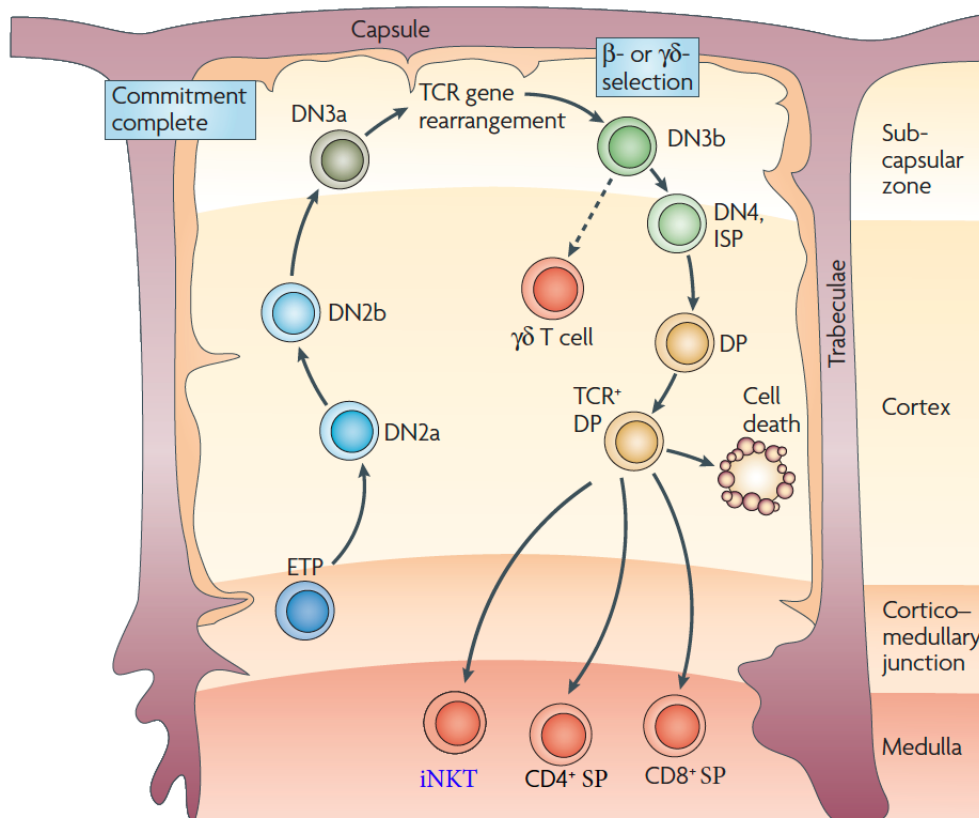


Figure 23. Stages in early T cell development. Cross-section of an adult thymic lobule showing the migration path of T-cell precursors. The early T cell precursors (ETPs) initially enter the thymus through blood vessels near the cortico-medullary junction, and differentiate from DN to DP to SP stages or unconventional T cells like NKT cells through the distinct microenvironments of the thymus. β -selection occurs during the accumulation of DN3 T cells in the extreme outer portion of the thymus. ETP, early T-cell precursors; DN, double negative; DP, double positive; SP, single positive (CD4⁺ or CD8⁺ cells). **Adapted from Rothenberg et al. (2008).**

After positive selection, $\gamma\delta$ T cells can either leave the thymus and populate secondary lymphoid organs and blood, or further differentiate into dendritic epidermal $\gamma\delta$ cells (DETCs) ([Asarnow et al., 1988](#)), IL-17A-producing $\gamma\delta$ cells ($\gamma\delta$ 17 cells) ([Haas et al., 2009](#)) or NK1.1⁺ $\gamma\delta$ cells ($\gamma\delta$ NKT cells) in the thymus ([Arase et al., 1995](#)). The use of PLZF and NK1.1 enables to discriminate two distinct $\gamma\delta$ T cells in the thymus: PLZF^{low}NK1.1⁺ and PLZF^{bright}NK1.1⁻ $\gamma\delta$ T cells ([Pereira and Boucontet, 2012](#)). The TF T-helper inducing POZ-kruppel liker factor (ThPOK) is required for the development of NK1.1⁺ $\gamma\delta$ T cells along with PLZF ([Alonzo et al., 2010](#)) and the negative regulator Tec family tyrosine kinase Itk ([Felices et al., 2009](#)). Interestingly, Styk1 is exclusively expressed in lymphocytes that are NK1.1-positive including NK

cells, $\alpha\beta$ iNKT cells, and $\gamma\delta$ NKT cells, but is dispensable for their development and function ([Wilharm et al., 2019](#); [Fauteux-Daniel et al., 2019](#)).

Upon activation, NK1.1⁺ $\gamma\delta$ T cells produce more IFN- γ than IL-4, whereas NK1.1⁻ $\gamma\delta$ T cells secrete more IL-4 than IFN- γ , which is reminiscent of the previously described NK1.1⁺ and NK1.1⁻ NKT cells ([Pereira and Boucontet, 2012](#)). And the capacity of $\gamma\delta$ T cells to produce cytokines occurs earlier than $\alpha\beta$ T cells ([Ferrick et al., 1995](#)). In fact, $\alpha\beta$ and $\gamma\delta$ T cells share great similarities regarding the development and functions, and compete for the same thymic niches ([Pereira and Boucontet, 2012](#); [Felices et al., 2009](#)). NK1.1⁺ $\gamma\delta$ T cells were reportedly involved in immunosurveillance as this population expands dramatically in early tumors in an IL-15-dependent way, with higher expression of CD49a and CD103 ([Dadi et al., 2016](#)). This protective effect could be attributed to their ability to sense tumor-associated signals via NKG2D which is constitutively expressed by NK1.1⁺ $\gamma\delta$ T cells ([Dadi et al., 2016](#)).

4 Research projects

4.1 Scientific context and objectives

TFs orchestrate cell fate and lineage development by controlling gene expression in time and space. During my thesis, I studied the role of several of them in the transcriptional control of either NK cell or iNKT cell fate. This report is divided into two parts, accordingly.

Project 1 aimed at understanding the role of T-bet and Eomes in NK cell development and maturation. While a growing number of key TFs are identified in the field of cytotoxic cells, the molecular mechanisms establishing their functions are poorly understood, and this remains the case for T-bet and Eomes that have been identified in this field for decades. T-bet and Eomes belong to T-box transcription factor family and are both expressed in conventional NK cells, albeit at different kinetics. The T-box DNA binding domains of T-bet and Eomes present a strong degree of identity, suggesting that they bind to the same DNA sequences. By contrast, the sequence homology of other portions of both proteins is quite low, suggesting that their interacting partners are distinct. Whether T-bet and Eomes have redundant, complementary or antagonistic activities on gene transcription remains an unanswered question, of central importance for the understanding of NK cell differentiation. To address this issue, we wanted to:

- 1) Measure the expression levels and define the subcellular localization of T-bet and Eomes during NK cell maturation in BM and spleen;
- 2) Analyze the role of T-bet and Eomes in NK cell development and maturation in a careful way by excluding ILC1 cells using additional markers CD49a and CD49b;
- 3) Identify genes regulated by either T-bet or Eomes by conducting transcriptomic analysis;
- 4) Map T-bet and Eomes binding sites in the genome in order to identify their target genes in NK cells;

5) Analyze the difference of chromatin accessibility in the absence of either T-bet or Eomes using ATAC-seq and understand the role of T-bet and Eomes in the regulation of chromatin accessibility during NK cell maturation.

Project 2 intended to clarify the cellular and molecular mechanism of Zeb1 in iNKT cell development. Zeb1 belongs to the Zeb family of TFs and is best known for its role in epithelial-to-mesenchymal transition (EMT). My team had previously shown an important role of Zeb2 in NK cell development. Moreover, Bruce Butler's group showed that *Cellophane* mutant mice that express a truncated form of Zeb1 have a decreased number of spleen NK cells. Based on this observation, my team started working on NK cell development in these mice, but found that the role of Zeb1 in NK cell development was likely cell extrinsic (Dylan Cherrier, Cécile Daussy and coworkers, unpublished observations). In the course of these studies, preliminary observations suggested a role of Zeb1 in iNKT cell development. In addition, previous descriptive studies have reported developmental defects of T cells in *Zeb1*^{-/-} and Zeb1 mutant mice with a deletion of C-terminal zinc finger region (Takagi et al., 1998; Higashi et al., 1997). We therefore set the following objectives:

- 1) Perform general phenotyping of immune subsets in *Cellophane* mice with particular focus on unconventional T cell subsets including iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49 expressing CD8 T cells;
- 2) Understand the potential mechanisms that result in a shrunked thymus in *Cellophane* mice (e.g. proliferation and survival);
- 3) Test if Zeb1 is involved in TCR signaling which is essential for T cell development and selection;
- 4) Conduct transcriptomic analysis on CD4⁺CD8⁺ DP cells to shed light on the underlying mechanism.

4.2 Project 1: T-bet and Eomes in NK cell development

4.2.1 Article 1

Sequential actions of Eomes and T-bet promote stepwise maturation of Natural Killer cells

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In preparation

Sequential actions of Eomes and T-bet promote stepwise maturation of Natural Killer cells

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Abstract

Eomes and T-bet are T-box transcription factors (TFs) co-expressed in NK cells, which regulate their development. The specific actions of each factor remain poorly understood. Here, we demonstrate that Eomes and T-bet have complementary roles in NK cells and regulate largely distinct gene sets. Eomes is dominantly expressed in immature NK cells and drives early lineage specification by inducing hallmark genes and functions. By contrast, T-bet is dominant in mature NK cells and represses genes associated with cell cycle or with other lineages, indirectly through the induction of transcriptional repressors. Moreover, Eomes and T-bet have antagonistic effects on NK cell maturation, and their mutual repression equilibrates this process. Yet, many effector genes are also co-regulated by Eomes and T-bet, confirming their redundancy in certain aspects. Using novel transgenic mice facilitating T-bet and Eomes chromatin immunoprecipitation, we uncovered a strong overlap in DNA binding between Eomes and T-bet in NK cells and pointed to Runx3 as a cofactor of transcriptional regulation. A few sites preferentially bound by Eomes and T-bet may endow NK cells with specific function, ie cytotoxicity for Eomes and responsiveness to IL-12 for T-bet. However, in many cases, Eomes and T-bet likely bind the same regulatory elements in a sequential manner during maturation, and T-bet binding may either reinforce or suppress the effects of Eomes.

Introduction

Natural Killer (NK) cells are group 1 innate lymphoid cells (ILC) with an important role in antiviral¹ and anti-tumor responses². NK cells share many features with tissue-resident ILC1s, such as the responsiveness to IL-15, IL-12 and IL-18 cytokines, and the capacity to rapidly produce IFN- γ upon stimulation³. However, they differ from ILC1s by their capacity to circulate in the blood, by their expression of multiple receptors of the Ly49 family, and by their higher cytotoxic potential and by their expression of integrin subunits. In particular, CD49b and CD49a are expressed in a mutually exclusive manner by NK cells and ILC1s, respectively^{4,5}. Moreover, NK cells and ILC1s are developmentally distinct. Indeed, even though all ILCs share a common progenitor, NK cells rapidly branch out from the main ILC developmental pathway⁶, and the factors that promote this route remain unclear. NK cells then operate a process of maturation that starts in the bone marrow and continues in the periphery. This process includes at least three discrete stages that can be discriminated by surface expression levels of CD11b and CD27^{7,8}. CD11b⁻ CD27⁺ (hereafter referred as CD11b⁻ or stage 1) are the most immature NK cells, and give rise to CD11b⁺CD27⁺ (double positive, DP, stage 2) which then differentiate into CD11b⁺CD27⁻ (hereafter referred as CD27⁻ or stage 3). Mature NK cells (either DP or CD27⁻) are more cytotoxic than immature ones against tumor targets⁸ and express a distinct set of trafficking molecules that allow them to circulate in the blood. In particular, they express the sphingosine-1 phosphate receptor S1PR5, which promotes exit from lymphoid organs both in mouse and human^{9,10}.

The specification of immune lineages depends on a network of lineage-determining TFs that induce hallmark genes and repress the expression of other lineages, thereby restricting pluripotency as cells differentiate¹¹. NK cell development and maturation are orchestrated by a network of TFs including the related T-box TF T-bet and Eomes both in mouse and human^{12,13}. Eomes and T-bet are thought to have similar DNA binding properties, owing to their highly homologous T-box DNA binding domains, which are 74% identical. Large scale chromatin accessibility analysis across immune subtypes¹⁴⁻¹⁷ predicted a major role of T-box TFs in the regulation of NK-cell specific enhancers. However, they failed to discriminate between T-bet and Eomes in this role.

T-bet was cloned in 2000 and immediately recognized as an essential driver of Th1 differentiation and IFN- γ production in CD4 T cells¹⁸. A few years later, the analysis of T-bet deficient mice revealed an essential role for T-bet in NK cell homeostasis and function¹⁹. In

the absence of T-bet, NK cells display a higher turnover associated with higher apoptosis rate¹⁹, and an immature phenotype c-Kit^{high} CD43^{low}¹⁹, KLRG1⁻²⁰ and CD27⁺²¹. A more recent study suggested that T-bet stabilizes immature NK cell attributes, which somewhat contradicted previous findings²². As recognized later, however, this conclusion was reached after using an incorrect NK cell gating strategy that also included ILC1s⁴. T-bet is indeed essential for the development of ILC1s, whose phenotype is highly similar to that of immature NK cells⁴. Moreover, a recent single cell RNA-seq analysis that clearly discriminated between NK cells and other ILC subsets showed that T-bet suppresses the immature NK cell transcriptional signature, and proposed that it does so in an indirect way²³. Functionally, T-bet is important to promote NK cell responsiveness to IL-12¹⁹ and to support their blood circulation through the upregulation of *S1pr5*²⁴, which could be important for their capacity to control lung metastases in the B16 model^{25,26}. Additionally, T-bet promotes the survival of mouse cytomegalovirus-specific memory NK cells²⁷.

The function of Eomes was first studied in CD8⁺ T cells, where it was shown to promote the expression of the IL-15 receptor subunit Il2rβ (or CD122), together with T-bet²⁸. Therefore, Eomes and T-bet were proposed to redundantly regulate the differentiation of CD8⁺ effector T cells. Later *in vitro* studies showed a strong correlation between Eomes and Perforin expression in CD8⁺ T cells activated with antigen and cytokines^{29,30}. This led to the concept that Eomes rather than T-bet, drives the cytotoxic phenotype. Generation of floxed Eomes alleles allowed conditional deletion of Eomes in immune cells, circumventing the issue of lethality of Eomes knockout mice. Deletion of Eomes in Vav1⁺ immune cells²² or in NKp46⁺ cells³¹ leads to a substantial decrease in the number of NK1.1⁺ CD3⁻ cells, thus demonstrating the essentiality of Eomes for NK cell development. However, as discussed above, this subset includes not only NK cells but also the related ILC1s, thus, the precise phenotype of Eomes-deficient NK cells remained to be analyzed. A recent paper confirmed the essential role of Eomes in the development of the NK cell lineage using a Tamoxifen-inducible Eomes deletion system. They found that Eomes was required to preserve NK cell viability, especially at the CD27⁺CD11b⁺ stage, and that it was essential for cytotoxicity but not for IFN-γ secretion³². How Eomes promotes NK cell development ie what genes are specifically regulated by this TF remains however unclear.

The nature of Eomes and T-bet cooperation is another unresolved question. Do they play redundant and additive roles or do they have specific and complementary functions or even

antagonistic ones at some loci? Overexpression of Eomes rescues IFN- γ production by T-bet deficient CD4⁺ T cells^{33,34} and promotes cytotoxicity in CD4⁺ T cells. Similar to T-bet, Eomes can also repress IL-5 production in memory Th2 cells³⁵. Therefore, most of the defined roles for Eomes in CD4⁺ T cells seem to be redundant with T-bet when they are overexpressed *in vitro*. Moreover, the combined deletion of T-bet and Eomes leads to a complete deficiency in NK cells while single mutations of either gene leads to a partial defect²². Together with the similarity in Eomes and T-bet DNA binding domains, this observation argues for a redundancy between both factors during NK cell development. Yet, the direct and indirect Eomes targets in NK cells are mostly unknown, as only a few studies have assessed their DNA binding properties (for a review see reference 36), but not in resting NK cells, and not in a comparative manner with T-bet. CHIP-seq analyses would represent a significant advance. However, such analyses remain undeniably complicated in primary cells and the paucity of appropriate tools and reagents to assess T-bet and Eomes binding at the genomic level are a further technical challenge. Thus, the cellular and molecular roles of T-bet and Eomes during NK cell development and maturation remain to be clarified.

Here we present a comprehensive analysis of the role of Eomes and T-bet during NK cell development. We showed a dominant expression and a dominant role of Eomes in immature NK cells and reciprocally of T-bet in mature ones. Eomes was required to specify the NK cell lineage and promote the survival of immature NK cells while T-bet was required for terminal differentiation but was not involved in early development. This division of labor was also visible when we compared the gene expression profile of immature and mature NK cells in the presence or absence of Eomes or T-bet. Eomes acted more often as an activator of transcription inducing the expression of many NK cell signature genes such as NK cell receptors at the immature stage, while T-bet acted more frequently as a repressor decreasing the expression of many T cell or progenitor genes at the mature stage. The latter activity was associated with T-bet-specific ability to induce transcriptional repressors such as Zeb2 and Blimp1. Using newly generated mouse models expressing endogenously tagged T-bet and Eomes, we performed genome-wide analysis of T-bet and Eomes binding. This comprehensive analysis showed that Eomes and T-bet binding sites are largely overlapping, suggesting a switch between Eomes and T-bet in the regulation of many NK cell genes during maturation.

Results

Eomes and T-bet balance each other during NK cell maturation and activation

To explore the roles of T-bet and Eomes in NK cell maturation, we first measured the expression of both TFs during maturation. To unambiguously analyze NK cells and exclude ILC1s we defined NK cells as CD49b⁺CD49a⁻NK1.1⁺CD3⁻ (Figure S1A) and analyzed maturation based on the CD11b/CD27 classification. Flow cytometry measurements indicated that Eomes expression in spleen WT NK cells was rather similar in stages 1-2 and then decreased in stage 3 NK cells, while T-bet had the reciprocal expression pattern (Figure 1A). In the BM, Eomes levels showed the same pattern as in the spleen while T-bet levels were much decreased compared to spleen NK cells (Figure S1B), as we previously observed⁴. In T-bet deficient NK cells (ie from *Tbx21*^{-/-} mice), a higher expression of Eomes was noted in all subsets, while Eomes-deficient NK cells (ie from *NCR1-iCre*^{+/-} *Eomes*^{fl/fl} mice, referred to as *NK-Eomes*^{-/-} thereafter) displayed higher T-bet expression (Figure 1A), showing that Eomes and T-bet repress each other in NK cells. The analysis of mice with a single copy of T-bet or Eomes suggested a dose-dependent effect of both TF on the repression of each other. Upon stimulation with cytokines ex vivo, IL-15 maintained T-bet and Eomes levels while a combination of IL-12 and IL-18 (together or not with IL-15) resulted in a strong up regulation of T-bet and a decrease of Eomes, further supporting the concept that Eomes and T-bet balance each other during NK cell maturation or activation (Figure 1B).

A previous article reported that T-bet and Eomes were localized both in the nucleus and in the cytoplasm of memory CD8⁺ T cells³⁶, which adds another layer of regulation for both TF. To analyze the nuclear expression of Eomes and T-bet, we used Image cytometry (Image-Stream) and confocal microscopy. Image-Stream analysis allowed us to quantify the nuclear fraction of Eomes and T-bet, delimited by the DAPI staining (Figure 1C). Using this method, we found that NK cell maturation was associated with a progressive shift in the ratio between Eomes and T-bet, with nuclear Eomes being dominant in immature NK cells and nuclear T-bet prevailing in mature NK cells (Figure 1D). A confocal microscopy analysis then showed that Eomes and T-bet are predominantly nuclear in NK cells, and indicated that they are localized in the same nuclear areas, rather excluded from DAPI-dense regions corresponding to heterochromatin, and this both in immature (Figure 1E) and mature NK cells (Figure S1B). Thus, Eomes and T-bet nuclear levels change in opposite ways during NK

cell maturation but the nuclear distribution of both TFs remains comparable during this process.

Eomes is essential for early NK cell development and restrains T-bet-induced terminal maturation

A previous analysis of the role of T-bet and Eomes in NK cell development was hampered by a gating analysis that did not discriminate between NK cells and ILC1²². We therefore revisited the role of both TFs in NK cell development and maturation using *Tbx21*^{-/-}, *NK-Eomes*^{-/-} and appropriate control mice by carefully gating NK cells and excluding ILC1 from the analysis (see Figure S1). Both T-bet and Eomes were important for NK cell homeostasis. However, Eomes had a more important role than T-bet in terms of NK cell numbers, ie the lack of Eomes caused the number of NK cells to drop by 5-10 fold both in BM and spleen, while the lack of T-bet rather increased the number of BM NK cells, and decreased that of spleen NK cells two to threefold (Figure 2A-B). When examining maturation stages, Eomes was essential for the accumulation of all NK cell subsets, especially DP that were the only ones to decrease in percentage among total NK cells in both BM and spleen compared to controls (Figure 2C). The strong decrease in the number of immature CD11b⁻ NK cells in the BM in the absence of Eomes indicated an essential role of this TF in early NK cell development, unlike T-bet. Of note, the paucity of NK cells in *NK-Eomes*^{-/-} mice was not compensated by an accumulation of CD49a⁺ ILC1 either CD49b⁺ or CD49b⁻ (Figure S2A).

T-bet deficiency resulted in an accumulation of DP both in terms of percentage and number in the BM and a near lack of CD27⁻ cells in both organs analyzed (Figure 2C), as previously shown^{4,21}, confirming the essential role of this TF in terminal NK cell maturation. We also observed an inverse pattern of KLRG1 expression in NK cell subsets in the absence of Eomes and T-bet, ie an increased percentage of KLRG1⁺ NK cells and a decreased percentage of these cells among NK cell subsets when compared to controls respectively, suggesting that terminal maturation is promoted by T-bet and rather prevented by Eomes (Figure S2B). To further test this point we sorted NK cells at maturation stages 1 and 2 from *Tbx21*^{-/-}, *NK-Eomes*^{-/-} and control mice and adoptively transferred them into un-irradiated Ly5a X B6 (CD45.1/2) mice (Figure 2D). Transferred NK cells were allowed to acclimate for two weeks before analysis of their maturation status in the spleen. As shown in Figure 2D, *NK-Eomes*^{-/-} NK cells from either stage 1 or stage 2 had an accelerated maturation towards the CD27-

CD11b⁺ stage 3 compared to controls, while *Tbx21*^{-/-} NK cells had the opposite behavior, thus confirming the antagonistic effect of both TF on NK cell maturation rate. Collectively, these results show that Eomes is essential to promote early NK cell development and to mitigate the pro-maturation effect of T-bet.

Eomes and T-bet limit proliferation and promote NK cell survival at different maturation stages

To further document the role of Eomes and T-bet in NK cell development and homeostasis, we next assessed proliferation and survival of NK cell subsets developing in the absence of either factor. We evaluated NK cell proliferation using EdU incorporation or Ki67 staining. The main proliferative burst in NK cells normally occurs before the acquisition of the CD11b integrin, in the bone marrow³⁷. Surprisingly, Eomes deletion did not change the level of proliferation of immature CD11b⁻ NK cells in the BM, but rather increased that of DP in this compartment. In the spleen, all *NK-Eomes*^{-/-} NK cell subsets had an increased proliferation rate, especially CD11b⁻ and DP (Figure 3A-B). T-bet deletion increased the proliferation rate of mature CD27⁻ NK cells and to a lesser extent that of DP, both in the BM and spleen, while CD11b⁻ NK cells were hardly affected. Thus, Eomes and T-bet have complementary roles in limiting proliferation of NK cell subsets at immature and mature stages respectively. As this increased turnover did not explain the reduction in NK cells upon deletion of either factor, we hypothesized that both TFs could regulate their survival. Indeed, the percentage of apoptotic Annexin-V⁺ CD11b⁻ and DP cells was increased in the absence of Eomes in the BM. Similarly, the percentage of apoptotic Annexin-V⁺ CD27⁻ NK cells was increased in the absence of T-bet in both BM and spleen (Figure 3C). As IL-15 is a major mediator of NK cell survival³⁸, we next assessed how Eomes and T-bet regulated the response to this cytokine. Upon co-culture with IL-15 for 48h, both Eomes and T-bet deficient NK cells showed a decreased viability compared to controls (Figure 3D) suggesting that a balanced expression of Eomes and T-bet is necessary for optimal response to IL-15.

Altogether, these data demonstrate that Eomes and T-bet have complementary roles in NK cell homeostasis by repressing proliferation and promoting survival at immature vs mature stages respectively, which could involve fine tuning of IL-15 responsiveness.

Complementary roles of Eomes and T-bet in instructing the NK cell maturation program

Next, to gain molecular insight on the mechanisms of T-bet and Eomes action during NK cell development and maturation, we performed RNA-seq in immature CD11b⁻ and mature CD27⁻ NK cells from *Tbx21*^{-/-}, *NK-Eomes*^{-/-} and control mice. In accordance with the dominant expression of Eomes in immature cells (Figure 1), we found that the loss of Eomes but not that of T-bet had a strong impact on gene expression in immature NK cells (Figure 4A-B, and table S1). Inversely, in mature NK cells, T-bet had a much more prominent role than Eomes on gene transcription (Figure 4A-B). When examining differentially expressed genes (DEGs), Eomes was more often a transactivator of gene expression (84% of DEG in immature NK cells and 52% in mature ones) while T-bet was more often a repressor of gene expression (63% of DEG in mature NK cells, Figure 4B). We queried the Immgen database³⁹ to visualize the expression pattern of Eomes and T-bet-regulated genes across the whole immune system. Eomes-induced genes were found to be rather NK-specific while T-bet activated genes had a broader expression pattern (Figure S3A). The expression pattern of Eomes-repressed and T-bet-repressed genes was more comparable, but T-bet appeared more specialized in the repression of genes expressed in hematopoietic progenitors, or in B and T cell progenitors (Figure S3B). Of note, Eomes-dependent genes in immature NK cells include 23/91 of genes defining the NK cell signature as previously defined by the Immgen consortium^{39,40}, while only four genes were also regulated by T-bet in this list (Figure 4C). Inversely, in mature NK cells, 25 and 6 of these genes were regulated by T-bet and Eomes respectively, suggesting that both Eomes and T-bet contribute to define NK cell identity, but in a sequential manner. Overall, there was a limited overlap between T-bet and Eomes dependent genes both in immature and mature NK cells (Figure 4D, Table S1), with 114/166 (68%) Eomes dependent genes not regulated by T-bet and 691/744 (92%) T-bet dependent genes not regulated by Eomes, thus showing that both TFs regulate transcription in a way that is to a large extent independent of each other. Finally, 82/166 Eomes-dependent (52%) and 250/744 T-bet-dependent (35%) genes were regulated during NK cell maturation (Figure 4E-F). Reciprocally, 294/699 genes normally regulated during NK cell maturation were dependent on either Eomes or T-bet (42%) (Figure 4F), confirming that both factors are major drivers of NK cell maturation, acting in a complementary manner.

Specific and shared actions of T-bet and Eomes on NK cell transcriptome

To define which properties were commonly or specifically conferred to NK cells by Eomes and T-bet, we then performed functional annotations of the gene modules described in Figure 4.

T-bet/Eomes co-regulated genes (53)

Only one gene was regulated together by T-bet and Eomes in both immature and mature NK cells: *Cym*, which encodes a mast cell enzyme of unknown function in NK cells (Figure 4D). Fifty-two other genes were regulated both by T-bet and Eomes in at least one comparison (Figure 4D). This gene module is presented in details in Figure 5 and includes three subsets: genes repressed by both TF (Figure 5A-B), genes induced by both T-bet and Eomes (Figure 5C-E) or genes induced by Eomes but repressed by T-bet (Figure 5G-I).

T-bet/Eomes-co-repressed genes (Figure 5A) notably include a cluster of genes involved in cell cycle (*Top2a*, *CCna2* etc), and *IL7R*, a hallmark gene of lymphoid progenitors and ILCs. *IL7R* expression was also higher at the protein level in both *Tbx21*^{-/-} and *NK-Eomes*^{-/-} NK cells compared to controls (Figure 5B), and both *Tbx21*^{-/-} and *NK-Eomes*^{-/-} NK cells proliferated more than their WT counterparts in the spleen (Figure 3A-B).

T-bet/Eomes co-induced genes (Figure 5C) include many genes induced by Eomes in immature NK cells and by T-bet in mature ones, suggesting that a switch occurs between both factors at regulatory genomic sites during maturation. This group comprises crucial mediators of granule-dependent cytotoxicity such as *Prf1*, *Gzma* and *Serpnb9b*, and “cell killing” was one of the significant terms in a functional annotation of this geneset (Figure 5E) using Metascape⁴¹. At the protein level, we confirmed that *Gzma* was strongly reduced in both T-bet and Eomes deficient NK cells (Figure 5D). *Cma1* is another mast cell-protease present in granules that could also be involved in cytotoxicity. We assessed the cytotoxic potential of Eomes and T-bet deficient mature NK cells using a new technique adapted to low cell numbers⁴². T-bet deficient NK cells had a normal cytotoxicity, while Eomes deficient NK cells were poorly cytotoxic compared to controls (Figure 5F). The T-bet/Eomes co-induced gene set also included *S1pr5* that is essential for NK cells egress from the bone marrow^{10,43}. To examine how T-bet and Eomes contributed to this egress, we injected T-bet and Eomes deficient mice with anti-CD45.2 and sacrificed them 5 min later, thus brightly staining only cells that were in blood sinusoids. A reduced sinusoidal fraction was observed in T-bet but not in Eomes deficient mice (Figure S4), showing that even though both TFs regulate *S1pr5*, T-bet is the major factor promoting blood circulation of NK cells.

Several genes showed however opposite regulation by T-bet and Eomes (Figure 5G), for example CD27 and CD69 were induced by Eomes but repressed by T-bet in NK cells, which we could also confirm at the protein level (Figure 5H-I). These genes are normally expressed at high levels in immature NK cells and repressed in mature NK cells²⁶ suggesting that when T-bet is up regulated upon terminal maturation, it substitutes Eomes on the promoter of these genes and represses their expression. Other genes may be regulated in a reciprocal manner, ie repression by Eomes and induction by T-bet. This is the case of *Cd226* encoding for DNAM1, which expression was not statistically different between control and KOs at the mRNA level (data not shown), but was clearly different at the protein level (Figure 5J).

Eomes-specific genes (113)

Eomes specific genes (ie regulated either in immature or in mature NK cells by Eomes and not T-bet) include mainly Eomes-inducible ones (Figure 6A). A functional annotation of Eomes-activated genes suggested a link with NK cell activation, cytotoxicity and IFN γ production (Figure 6B). Indeed, this module contains a large cluster of NK cell receptors (KLRA8, KLRE1, KLRA4, KLRC2, KLRG1) and the signaling adaptor SAP (encoded by *Sh2d1a*), suggesting that Eomes is specifically involved in the induction of NK cell receptors and associated signaling and functions. Indeed, when looking at the expression of a battery of NK cell receptors at the protein level, we observed a defect in the expression of several Ly49 or SLAM receptors in immature NK cells deficient for Eomes (Figure 6C). This defect was usually not seen in more mature cells (data not shown), suggesting that other factors, such as T-bet could compensate for the lack of Eomes after maturation.

Eomes also activated the expression of the CD11b integrin (encoded by *Itgam*, Figure 6D), and genes involved in the cytotoxic function such as FasL and GzmK or in trafficking such as CD62L (encoded by *Sell*, Figure 6E) or the granule-contained CCL5 chemokine. Finally, Eomes induced the expression of many genes of unknown function such as the Kelch domain containing proteins KHDC1A and KHDC1B and several lncRNA (*Mirt1*, *Gm12596* etc, see table S1).

To identify putative downstream mediators of Eomes transcriptional activity, we also examined TFs whose expression was dependent on Eomes. We found that *Kcnip3*, *Lzts1* and *Bhlhe40* were down regulated in the absence of Eomes, either in immature or in mature NK cells (Figure 6F). *Bhlhe40* is known to promote mitochondrial metabolism in resident

memory T cells⁴⁴ and could therefore also regulate NK cell development. Eomes also behaved as a specific repressor of a subset of TFs including *Cebpg*, *Cbx2*, *Zfp689* and *Zfp683*. The latter, also known as Hobit, was previously defined as a master regulator of tissue residency⁴⁵. This suggested a contribution of Eomes to blood circulation by repressing Hobit at immature stages. However, data in Figure S4 show that T-bet is much more important than Eomes to promote the blood circulation phenotype in NK cells.

T-bet specific genes (692)

T-bet specific genes represent a large cluster of genes, with two thirds of them being repressed by T-bet and the other ones being induced by this TF (Figure 7A for the top 100 genes, and Table S1 for the complete list). A functional annotation of T-bet repressed genes retrieved “cell cycle” and related terms as the most significant ones (Figure 7B). “adaptive immunity”, “hematopoietic cell lineage”, “T cell activation” and “myeloid cell differentiation” were other significant terms, which reflects the ability of T-bet to repress progenitor/pluripotency genes or genes expressed in other immune lineages. For example, T-bet strongly repressed the expression of *TCF7* or that of CD3 subunits. *Csf2*, encoding for GM-CSF was also specifically repressed by T-bet and not changed by Eomes.

Gene repression could be direct or indirect via other TFs induced by T-bet. Indeed, the most significant functional term associated with T-bet induced genes was “negative regulation of gene expression, epigenetic”. Indeed, T-bet induced the expression of a large cluster of histone subunits (*Hist1h1c*, *Hist1h1e*, *Hist1h3a*) or histone methylation enzymes (*Phf1*, *Hdac5*, *Ezh1*, *Epc1*, Figure 7C). Moreover, T-bet specifically induced the expression of many transcriptional repressors (Figure 7C), some of which already known to regulate NK cell maturation such as *Zeb2*²⁶ and *Blimp* (encoded by *Prdm1*)⁴⁶, and some other like *Pogk* or *Sertad1* with no described role in NK cells.

The functional analysis of T-bet-induced genes (Figure 7B) also retrieved “IL-12 pathway” and “signaling by interleukins”, which reflects the ability of T-bet to endow NK cells with specific response to cytokines. In particular, T-bet induced the expression of IL-12 and IL-21 receptors. As a consequence, *Tbx21*^{-/-} NK cells had a defective ability to respond to IL-12 as measured by STAT4 phosphorylation (Figure 7D) and by IFN γ secretion (Figure 7E). Of note, IFN γ secretion by *Tbx21*^{-/-} NK cells was normal in response to tumor targets (data not shown). “Macroautophagy” was also associated with T-bet-induced genes (Figure 7B) while several

terms related to anabolic pathways were associated with T-bet-repressed genes. This correlates with the known role of autophagy in NK cell development and survival⁴⁷, and with the increased apoptosis observed in the absence of T-bet¹⁹ (Figure 3).

Eomes and T-bet share most of their DNA binding sites

To complement the RNA-seq analysis, we sought to identify T-bet and Eomes binding sites in NK cells. In an effort to decrease experimental variability and avoid a bias linked to the use of different antibodies, we generated two mouse models allowing ChIP-seq analysis of T-bet and Eomes using the same antibody. In these models, an HA-V5 encoding tag was inserted at the 3' end of T-bet and Eomes coding sequences (Figure 8A, see methods). Flow cytometry and WB analyses validated the expression of tagged T-bet and Eomes in NK cells even though HA-V5 tags decreased the expression of both Eomes and T-bet to some extent (Figure S5A-B). We then performed ChIP-seq of Eomes and T-bet using an HA antibody in freshly isolated NK cells.

We found similar numbers of binding sites for T-bet and Eomes (about 12000), and two thirds of these peaks were shared between T-bet and Eomes (Figure 8B) according to the ChIP-seq peak calling algorithm. The same proportions were kept when assigning genes to each peak (Figure 8C). This suggested that T-bet and Eomes compete for many of their binding sites. The overlap between T-bet and Eomes ChIP-seq was probably underestimated by the algorithm as judged by the visualization of selected "specific" Eomes and T-bet peaks. These peaks appear indeed in both ChIP-seq but with differential intensities (Figures S6 and S7). Both T-bet and Eomes were mainly bound to proximal (<1kb) regions relative to transcriptional start sites (TSS) (Figure 8D).

We then combined the ChIP-seq analysis with our RNA-seq results to identify direct T-bet and Eomes target genes. Genes associated with T-bet or Eomes binding were in general much more expressed than those without binding (Figure 8E), and co-binding did not influence further this expression. Eomes binding sites were discovered near 2944 genes expressed by NK cells (Figure 8F). Among these genes, 43 were differentially expressed between WT and *NK-Eomes*^{-/-} NK cells (Figure 8F), which included DEGs in both immature and mature NK cells (Figure S8, complete gene list in Table S2). This group of functional Eomes targets was in most cases induced by Eomes, and notably contains many genes

involved in cytotoxicity (*Gzma*, *Prf1*), NK cell receptors (*Klrc2*, *Klrb1b*), NK cell trafficking (*S1pr5*), chemo-attraction (*Ccl5*) and transcription (*Bhlhe40*).

T-bet binding sites were discovered near 2978 genes expressed by NK cells including 201 of the 734 genes differentially expressed between WT and *Tbx21*^{-/-} mature NK cells (Figure 8G, Figure S8 and Table S2). 181 of these peaks were also specifically bound by T-bet and notably include chemokines (*Ccl4*, *Xcl1*), TF (*Zeb2*), cytokines (*Csf2*), chemokine receptors (*Ccr5*, *Ccr7*) etc.

We also ranked genes based on the number of T-bet and Eomes peaks retrieved in the ChIP-seq analysis. Top genes in this list (Table S3) comprise many TFs, including known regulators of lymphocyte development *Fli1*, *Runx3* and T-bet/Eomes themselves. They also include genes of unknown functions in NK cells such as *Chsy1* or *Pik3ap1*. Corresponding ChIP-seq tracks are shown in Figure S9 and highlight the high degree of cross-regulation of T-bet and Eomes.

To identify putative T-bet and Eomes partners, we compared TF-binding motifs under T-bet and Eomes ChIP-seq peaks using the RSAT motif discovery tool⁴⁸. As expected the canonical T-box motif was enriched under Eomes peaks (Figure 8H), but surprisingly not under T-bet ones (Figure 8I). With this exception, the motif analysis yielded very similar results for T-bet and Eomes with a strong enrichment for Runx, Ets and Zinc-finger (ZF) motifs, in this order based on statistics. Since *Runx3* was previously shown to physically interact with Eomes⁴⁹, and T-box and *Runx3* were shown to cooperate in CTL development²⁹, it is possible that binding of T-bet and Eomes to DNA occurs very often in an indirect way through interaction with *Runx3* and possibly other TFs identified here.

Collectively, our ChIP-seq analysis uncovers a strong overlap in genomic DNA binding between Eomes and T-bet and highlights functional targets for both TFs as well as potential partners in regulatory complexes.

Discussion

Here, we provide molecular insight on how Eomes and T-bet control NK cell development and maturation. Using a variety of methods, we show that Eomes is dominantly expressed in immature NK cells and that deficiency in Eomes but not T-bet strongly affects the transcriptome of immature NK cells. In addition, we found that T-bet deficiency does not affect the number of immature NK cells, while Eomes deficiency resulted in a severe decrease of this subset and of other consecutive stages. Mechanistically, our data demonstrate that Eomes is essential to specify the NK cell lineage by inducing the expression of many hallmark genes important to provide survival signals (such as NK cell receptors⁵⁰) and by promoting optimal responsiveness to IL-15. In the absence of Eomes, immature NK cells were more apoptotic in the BM, confirming recent findings³². By contrast T-bet is dominantly expressed in mature NK cells, and is critical for terminal NK cell maturation, which confirms previous observations^{19–21,26}. We also found that Eomes and T-bet balance each other's expression, which is important to mitigate the maturation rate. Indeed, in the absence of Eomes, residual NK cells tend to differentiate much more quickly than control NK cells. Reciprocally, the absence of T-bet resulted in the accumulation of DP NK cells. Thus, the Eomes to T-bet ratio is a crucial rheostat of NK cell maturation. Subtle changes in this equilibrium could have important consequences on the representation of maturation subsets.

T-bet acted more often as a repressor in NK cells, suppressing the transcriptional program of immature NK cells. This could be linked to its ability to induce the expression of known repressors such as Zeb2²⁶ and Blimp1⁴⁶, and of many enzymes, histones subunits and complexes involved in epigenetic reprogramming, as shown in the present study. We previously described that Zeb2-deficient and T-bet-deficient NK cells were phenocopies, and that Zeb2 overexpression partly compensated for T-bet deficiency in NK cells²⁶. Altogether, this suggests that T-bet induction of transcriptional repressors such as Zeb2 is essential to suppress the expression of genes expressed at previous developmental stages ie pluripotency, progenitors or immature NK cells. Our study points to many potentially novel transcriptional regulators downstream of T-bet such as Pogk that contains a Krüppel-associated box domain (KRAB) repressor domain⁵¹, or Sertad1, a member of the Trip-Br family of TF, known to control cell proliferation⁵². The indirect effect of T-bet via

transcriptional repressors may also explain why there is overall a limited overlap between genes regulated by T-bet and Eomes, despite the fact that T-bet and Eomes DNA binding largely intersects. Interestingly, a previous analysis of Eomes and Brachyury actions in embryonic stem cells also showed that Eomes was rather involved in the induction of hallmark mesoderm genes, while Brachyury rather repressed neuroectoderm genes⁵³. Thus, division of labor between different T-box TFs may operate to favor differentiation in different tissues.

Eomes regulated the expression of very few TFs, with the notable exception of *Bhlhe40* and *Hobit*. *Bhlhe40* could be especially important for NK cell metabolism downstream of Eomes, as suggested by a recent study in memory CD8⁺ T cells⁴⁴. In this study, *Bhlhe40*^{-/-} memory T cells had a diminished expression of genes encoding the components of the mitochondrial membrane or genes involved in mitochondrial metabolism and/or OXPHOS, and a decreased oxygen consumption rate⁴⁴. *Hobit* has been previously reported to control tissue residency in lymphocytes, in cooperation with *Blimp1*⁴⁵. As NK cells are the only ILCs capable of circulating in the blood vasculature and the only ILCs to express Eomes, we hypothesized that Eomes promoted blood circulation by repressing *Hobit*. However, we found little impact of Eomes deficiency on the ability of residual NK cells to exit the BM or LN, as opposed to the effect of T-bet that was much pronounced, especially in mature NK cells. This effect could be mediated by *S1pr5*, which indubitably contributes to NK cell trafficking, as we previously showed^{10,43}. Why ILC1s are tissue resident and *S1pr5* negative despite high levels of T-bet remains to be determined. Additional factors, such as *Zeb2* that is induced at late stages of NK cell differentiation may be important to open the *S1pr5* locus.

T-bet was also necessary for the survival of mature CD27⁻ NK cells, which correlated with the T-bet dependent induction of the “macroautophagy” pathway as captured by the functional annotation of the T-bet dependent geneset. Autophagy is known to be absolutely essential for NK cells⁵⁴ and more generally for all ILC types⁴⁷. Glycolysis and oxidative phosphorylation are known to operate at very low intensity in NK cells and these pathways are even down regulated at the transcriptional level during NK cell differentiation⁵⁵. In this context, autophagy could be especially important to preserve NK cell integrity in quiescent mature NK cells. Autophagy is negatively regulated by the mTOR pathway, and we showed that activity of both mTORC1 and mTORC2 complexes was indeed progressively decreased during NK cell maturation⁵⁵. More recent studies have shown an interesting link between mTOR

complexes and T-box factors. Indeed, deletion of Raptor, the essential subunit of mTORC1 resulted in a specific decrease of Eomes expression while deletion of Rictor had the reciprocal effect on T-bet⁵⁶. The latter effect could be through the posttranscriptional regulation of Foxo1 that is known to negatively regulate T-bet expression in NK cells⁵⁷. How mTORC1 specifically regulates Eomes and how the mTORC1/mTORC2 equilibrium is controlled during maturation remain open questions.

We generated novel T-bet and Eomes knock-in mice with the same HA-V5 insertion at the C-terminus of each factor. We anticipate that such tools will be extremely useful to study the role of both TFs in different contexts. The study of epigenetic regulation is indeed hampered by the limited availability of antibodies against TFs, and by the low expression of many of them. Using these clean tools, we identified more than 12,000 peaks for both T-bet and Eomes in the resting NK cell genome, which exceeds previous attempts using T-bet antibodies^{15,58}. A very strong overlap between Eomes and T-bet binding was observed, which confirms a previous study in activated CD8⁺ T cells⁵⁹. However, the latter study used different antibodies for T-bet and Eomes, and *in vivo* activated CD8⁺ T cells vs *in vitro* activated CD8⁺ T cells as material for T-bet and Eomes ChIP-seq respectively, which could arguably lead to technical artifacts. Our study is thus the first comparison of genome-wide DNA binding by T-bet and Eomes using untouched primary lymphocytes. As the ChIP-seq signals are usually weak when working with endogenous levels of TFs, the “specificities” in T-bet or Eomes binding should be taken with caution, and more studies should be performed to formally prove that small differences in peak height really correspond to differential binding by T-bet and Eomes at selected sites.

Most T-bet and Eomes peaks are not associated with differential expression of the corresponding genes in T-bet or Eomes deficient NK cells, suggesting that other factors may compensate for the lack of T-bet or Eomes binding in most cases. This point is not specific of T-box TFs as similar conclusions have been reached for many other TFs, as previously reviewed⁶⁰. Thus, binding is not necessarily equal to regulation, and it is probable that only a small fraction of all binding events have an important impact on gene expression.

As the Eomes/T-bet balance switches during maturation from dominance of Eomes in immature NK cells to dominance of T-bet in mature NK cells, we propose that in many occurrences and for direct T-box targets, Eomes comes first and binds T-box motifs to initiate transcription of NK cell genes. Then, when the level of T-bet increases and that of

Eomes is repressed, T-bet would come and replace Eomes. While this model is technically challenging to demonstrate, it would explain why many genes such as *Prf1* or *GzmA* are apparently co-induced by Eomes and T-bet. T-bet binding could either have the same effect as Eomes on gene expression (cases of *Prf1*, *GzmA*, *S1pr5*, *IL7R*, etc), or alternatively have an opposed effect (cases of *CD27*, *CXCR3*, *CD69*, *DNAM1* etc). Opposed effects of T-bet and Eomes may involve secondary effectors as discussed above, or alternatively, different cofactors. Yet, our analysis of motifs associated with T-bet and Eomes peaks did not show important differences. In fact, several lines of evidence point to *Runx3* as a major cofactor of both T-bet and Eomes. This confirms previous observations of physical interactions between Eomes and *Runx3*⁴⁹ and of a cooperation between both T-bet and Eomes and *Runx3* during CD8⁺ T cell differentiation²⁹. Other cofactors may include members of the Ets family such as *Ets1*, which was previously shown to regulate NK cell development⁶¹ and Egr family members. *Runx3* and other factors may act as pioneer factors to activate enhancers in NK cell progenitors, as suggested by the observation that Eomes binds at sites previously bound by *Runx3* in memory CD8⁺ T cells⁴⁹. Binding of Eomes or T-bet at these *Runx3* sites may stabilize chromatin states, as previously suggested^{29,59}.

In conclusion, our study reveals major roles for Eomes in NK cell lineage specification and induction of NK cell hallmark genes such as NK cell receptors and genes associated with cytotoxicity. Reciprocally, we found that T-bet promotes terminal NK cell maturation and survival via the repression of lineage and pluripotency genes indirectly via the induction of different repressors and the induction of specific properties such as responsiveness to IL-12. T-bet and Eomes binding was largely overlapping, suggesting different modes of cooperation or antagonism between these factors, which likely depends on cofactors. Finally, different lines of evidence suggest that they are major regulators of chromatin accessibility, stabilizing the effect of other TFs such as *Runx3* during NK cell maturation.

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Figure legends

Figure 1. Eomes and T-bet balance each other during NK cell maturation. (A) Flow cytometry measurement of T-bet and Eomes in gated NK cells from WT, *Tbx21^{+/-}*, *Tbx21^{-/-}*, *Ncr1^{Cre/+}* (*Eomes^{+/+}*), *Ncr1^{Cre/+}* X *Eomes^{lox/+}* (*Eomes^{+/-}*) and *Ncr1^{Cre/+}* X *Eomes^{lox/lox}* (*Eomes^{-/-}*) mice, as indicated. Bar graphs show the mean +/-SD fluorescence intensity (MFI) of Eomes and T-bet staining in gated NK cell subsets from spleen. Data are representative of 3 experiments and 3 mice are shown for each group. **(B)** WT spleen cells were stimulated O/N in the indicated conditions and T-bet and Eomes expression were measured by flow cytometry. Data are representative of 2 experiments and 3 mice are shown for each group. **(C)** Image stream X (ISX) analysis of T-bet and Eomes expression in gated NK cells. Representative images are shown for the three NK cell subsets. CD122 staining was used to visualize the membrane. **(D)** Quantification of nuclear T-bet and Eomes in gated NK cell subsets analyzed with IDEAS software. Dots correspond to nuclear MFI of T-bet and Eomes for the indicated subsets. N=3 **(E)** NK cells were sorted from WT mice in immature or mature subsets and subsequently stained for nucleus (DAPI), T-bet, Eomes and CD122 for confocal microscopy analysis. Shown are representative images of each staining and combinations for immature NK cells. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 2. Eomes and T-bet promote NK cell survival and differentiation at different cellular transitions. (A) Flow cytometry analysis of NK cell percentage in BM or spleen lymphocytes of the indicated mice. Each dot corresponds to a single mouse (n=5-6, pooled from 2 experiments), and graphs show the mean percentage +/-SD. **(B)** Number of NK cells calculated from data in (A) combined with numeration of corresponding organs. **(C)** Proportion and numbers of NK cell subsets for the mice and organs analyzed in (A-B). **(D)** NK cells of the indicated subsets (CD11b⁻ or DP) and genotypes were FACS-sorted and then adoptively transferred into congenic unirradiated Ly5a X B6 mice (CD45.1/2). Two weeks later, spleen NK cells were purified from these mice and the CD11b/CD27 phenotype of transferred NK cells (identified by their CD45.2 expression) was analyzed by flow cytometry. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 3: Eomes and T-bet regulate NK cell proliferation and survival at different stages. (A) Mice were injected twice with EdU within a two-day-period, and EdU incorporation was measured in NK cells from the indicated organs by flow cytometry one day after the last injection. Each dot corresponds to a single mouse (n=6, pooled from 2 experiments), and graphs show the mean percentage +/-SD. **(B)** Flow cytometry analysis of Ki67 expression in BM and spleen NK cells. Each dot corresponds to a single mouse (n=6, pooled from 2 experiments), and graphs show the mean percentage +/-SD. **(C)** *Ex vivo* apoptosis of BM and spleen NK cell subsets as assessed by flow cytometry analysis of Annexin-V staining. Bar graphs show the mean percentage +/-SD of Annexin-V+ cells within each subset, dots correspond to individual mice analyzed. **(D)** Total NK cell viability (% Annexin-V negative cells) after culture of splenocytes with IL-15 for 48h at the indicated concentrations. Data are from 3 mice representative of 2 independent experiments. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 4. Eomes and T-bet have mostly distinct roles on NK cell transcription during maturation. RNA-seq analysis of sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control CD11b⁻ and CD27⁻ NK cells (n=3 per group with three sorts in total). DEG were selected based on adjusted p-value<0.05. **(A)** Volcano plots of gene expression in immature CD11b⁻ or mature CD27⁻ NK cells comparing T-bet or Eomes deficient cells with appropriate controls. Selected genes are highlighted with their names. **(B)** Bar histograms showing the number of genes induced or repressed by T-bet or Eomes in immature and mature NK cells. **(C)** Venn diagram showing the overlap between the Immgen-defined NK cell signature and the Eomes-activated and T-bet-activated genes in immature and mature NK cells. **(D)** Venn diagram showing the overlap between T-bet regulated and Eomes-regulated genes in immature and mature NK cells. **(E)** Volcano plots comparing the expression of T-bet or Eomes dependent genes between WT immature and mature NK cells. Selected genes are highlighted with their names. **(F)** Venn diagram showing the overlap between T-bet-dependent, Eomes-dependent and maturation-regulated genes.

Figure 5. Shared transcriptional activities of T-bet and Eomes in NK cells. RNA-seq analysis of sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control CD11b⁻ and CD27⁻ NK cells (n=3 per group with three sorts in total). DEGs were selected based on adjusted p-value<0.05. **(A)** T-bet/Eomes co-repressed genes in immature or mature NK cells. Bar graphs show the log₂ transformed fold change of genes between controls and T-bet or Eomes deficient mice, as indicated. **(B)** Bar graph showing the mean±SD of IL7R expression in NK cells of the indicated genotype as measured by flow cytometry. The different controls for each genotype are pooled and annotated as “WT” in the graph which is same for the following graphs in (D, H-J). N=3. **(C)** T-bet/Eomes co-induced genes in immature or mature NK cells, Bar graphs show the log₂ transformed fold change of genes between controls and T-bet or Eomes deficient mice, as indicated. **(D)** Bar graph showing the mean±SD of GzmA expression in NK cells of the indicated genotype as measured by flow cytometry. N=3 **(E)** Functional annotation of the T-bet/Eomes induced gene set using Metascape. Bar graphs show selected terms among the most significant ones. **(F)** Cytotoxicity assay using sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control CD27⁻ NK cells as effectors and RMA-KR-Nano-luc cells as targets. **(G)** Bar graph showing the log₂ transformed fold change of genes regulated in opposite ways by T-bet and Eomes, between controls and T-bet or Eomes deficient mice, as indicated. **(H-J)** Bar graphs showing the mean±SD of CD27 (H), CD69 (I) and DNAM-1 (J) expression in NK cells of the indicated genotype as measured by flow cytometry. N=3. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 6. Eomes-specific role in the induction of hallmark NK cell genes. RNA-seq analysis of sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control CD11b⁻ and CD27⁻ NK cells (N=3 per group with three sorts in total). DEGs were selected based on adjusted p-value<0.05. **(A)** Genes specifically regulated by Eomes in immature or mature NK cells. Bar graph show the log₂ transformed fold change between controls and Eomes deficient NK cells, as indicated. A few selected gene names are shown. **(B)** Functional annotation of the Eomes induced gene set using Metascape. Bar graphs show selected terms among the most significant ones. **(C-E)** Overlaid FACS histograms showing the expression of the indicated surface molecules in Eomes deficient vs control NK cells, as indicated. Grey histograms show control staining. **(F)**

Heatmaps showing the log₂ transformed fold change in the expression of the indicated TF between control and Eomes deficient NK cells.

Figure 7. T-bet-specific role in the repression of pluripotency and cell proliferation. RNA-seq analysis of sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control CD11b⁻ and CD27⁻ NK cells (n=3 per group with three sorts in total). DEGs were selected based on adjusted p-value<0.05. **(A)** Genes specifically regulated by T-bet in mature CD27⁻ NK cells. Bar graphs show the log₂ transformed fold change between controls and T-bet deficient mice, as indicated. A few selected gene names are shown. **(B)** Functional annotation of the T-bet-repressed and T-bet-induced genesets using Metascape. Bar graphs show selected terms among the most significant ones. **(C)** Transcription factors, histone subunits or histone modifying enzymes whose expression are dependent on T-bet. The heatmap shows the log₂ transformed fold change in expression between control and T-bet deficient mature NK cells. **(D)** Flow cytometry analysis of STAT4 phosphorylation in WT vs T-bet deficient NK cells of the indicated subset in response to stimulation with IL-12 for 1h. N=3. **(E)** Flow cytometry analysis of intracellular IFN γ expression in gated spleen NK cells of the indicated genotype following culture in medium supplemented or not with IL-12 and IL-18. N=6 mice in 2 experiments. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 8. Identification of direct T-bet and Eomes targets using novel KI mice. **(A)** Scheme of knock-in Eomes and T-bet HAV5 constructs. **(B)** Venn diagram showing the overlap between Eomes and T-bet CHIP-seq peaks. **(C)** Venn diagram showing the overlap between genes near Eomes and T-bet peaks. **(D)** Plot showing the distribution of T-bet and Eomes peaks in the mouse genome based on the distance to TSS. **(E)** Histogram of mean transcript expression with respect to T-bet and/or Eomes binding according to RNA-seq data. **(F)** Venn diagram showing the overlap between Eomes-bound (CHIP-seq) and Eomes-regulated genes (RNA-seq). **(G)** Venn diagram showing the overlap between T-bet-bound (CHIP-seq) and T-bet-regulated genes (RNA-seq). **(H-I)** Motifs associated with (H) Eomes or (I) T-bet peaks and corresponding TFs ranked by significance.

Supplementary figures and tables

Table S1. Genes differentially expressed in *Tbx21*^{-/-}, or *NK-Eomes*^{-/-} immature or mature NK cells compared to controls. The list is ranked from top to bottom based on the number of comparisons for which differential expression was measured. Log2 fold changes and adjusted p-value for the comparisons are shown.

Table S2. List of validated T-bet and Eomes target genes, ie genes for which DNA binding was measured for T-bet or Eomes, and for which differential expression was measured in *Tbx21*^{-/-}, or *NK-Eomes*^{-/-} immature or mature NK cells compared to controls, respectively.

Table S3. List of 30 top genes ranked on the basis of the number of T-bet and Eomes CHIP-seq peaks associated to each gene. Genes encoding for TFs are highlighted in yellow.

Table S4. List of genes overlapped between Eomes- or T-bet-bound (CHIP-seq) and Eomes- or T-bet-regulated genes (RNA-seq).

Figure S1. Extended data for Figure 1. (A) Gating strategy of NK cells in this study. NK cells were defined as NK1.1+CD3-CD49b+CD49a-. NK cell subsets CD11b-, DP and CD27- were gated as shown in the right panel. **(B)** Flow cytometry measurement of T-bet and Eomes in gated NK cells from WT, *Tbx21*^{+/-}, *Tbx21*^{-/-}, *Ncr1*^{Cre/+} (*Eomes*^{+/+}), *Ncr1*^{Cre/+} X *Eomes*^{lox/+} (*Eomes*^{+/-}) and *Ncr1*^{Cre/+} X *Eomes*^{lox/lox} (*Eomes*^{-/-}) mice, as indicated. Bar graphs show the mean +/-SD fluorescence intensity (MFI) of Eomes and T-bet staining in gated NK cell subsets from the BM. Data are representative of 3 experiments and 3 mice are shown for each group. **(C)** NK cells were sorted from WT mice in immature or mature subsets and subsequently stained for nucleus (DAPI), T-bet, Eomes and CD122 for confocal microscopy analysis. Shown are representative photographs of each staining and combinations for mature NK cells. Data are representative of 2 experiments.

Figure S2. Extended data for Figure 2.

(A) Number of NK1.1+CD3- ILCs of the CD49a+ CD49b-, CD49a+CD49b+, CD49a-CD49b+ and CD49a-CD49b- phenotype in the indicated organs of WT, *NK-Eomes*^{-/-} and *Tbx21*^{-/-} mice. (B) FACS measurement of KLRG1 expression in gated spleen NK cells from mice of the indicated genotype. Bar graphs show the mean and dots corresponding to individual mice. Data are

polled from 2 experiments with n=4-6 mice in total. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure S3. Expression pattern of NK cell genes regulated by T-bet or Eomes across the immune system. (A) Expression profile of NK cell genes activated by Eomes or T-bet across immune subsets (Immgen data³⁹). **(B)** Expression profile of NK cell genes repressed by Eomes or T-bet across immune subsets (Immgen data³⁹).

Figure S4. T-bet promotes NK cell blood circulation. Mice of the indicated genotypes were injected with CD45 MAb intravenously 5 minutes before sacrifice. The percentages of CD45+ NK cells among different subsets of different organs were analyzed by flow cytometry. Results were pooled from N=2 experiments with N=4-6 mice in total. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure S5. Validation of HA-V5 expression in knock-in mice. (A) Flow cytometry analysis of T-bet, Eomes, and HA expression in gated NK cells from the indicated mouse strains. **(B)** WB analysis of HA and V5 expression in splenocytes from the indicated mouse strains.

Figure S6. Selected examples of Eomes ChIP-seq tracks for validated functional Eomes targets. Shaded areas correspond to “specific” Eomes peaks.

Figure S7. Selected examples of T-bet ChIP-seq tracks for validated functional T-bet targets. Shaded areas correspond to “specific” T-bet peaks.

Figure S8. Overlap between ChIP-seq and DEGs in immature and mature NK cells. (A) Venn diagram showing the overlap between Eomes binding (ChIP-seq) and genes significantly regulated by Eomes in immature or mature NK cells (RNA-seq, p-value<0.05). **(B)** Venn diagram showing the overlap between T-bet binding (ChIP-seq) and genes significantly regulated by T-bet in immature or mature NK cells (RNA-seq, p-value<0.05).

Figure S9. ChIP-seq tracks for genes with a high number of T-bet and Eomes peaks.

Material and Methods

Mice

Wild-type C57BL/6 mice were purchased from Charles River Laboratories (L'Arbresle). *NCR1-iCre*⁶², *Eomes*^{lox/lox}⁶³ and *Tbx21*^{-/-}¹⁸ mice were previously described. This study was carried out in accordance with the French recommendations in the Guide for the ethical evaluation of experiments using laboratory animals and the European guidelines 86/609/CEE. The bioethic local committee CECCAPP approved all experimental studies. Mice were bred in the Plateau de Biologie Expérimentale de la Souris, our animal facility.

Flow cytometry

Single-cell suspensions of mouse spleen, liver and bone marrow were obtained and stained. Intracellular stainings for TFs were performed using Foxp3 kit (ebioscience). Cell viability was measured using Annexin-V (BD Biosciences)/live-dead fixable (eBiosciences) staining. Lyse/Fix and PermIII buffers (BD Biosciences) were used for intracellular staining of phosphorylated proteins. Flow cytometry was carried out on a FACS LSR II, or a FACS Fortessa (Becton-Dickinson). Data were analysed using FlowJo (Treestar). Antibodies were purchased from eBioscience, BD biosciences, R&D Systems, Abcam, Beckman-Coulter, Miltenyi or Biolegend. We used the following antibodies: anti-CD3 (clone 145-2C11), anti-CD19 (clone 1D3), anti-NK1.1 (clone PK136), anti-NKp46 (clone 29A1.4), anti-CD11b (clone M1/70), anti-CD27 (clone LG.7F9), anti-CD49a (clone HA31/8), anti-CD49b (clone DX5), anti-CD122 (clone SH4), anti-CD127 (clone A7R34), anti-CD62L (clone MEL-14), anti-Ly49A (clone YE1/48.10.6), anti-Ly49D (clone 4E5), anti-Ly49E/F (clone REA218), anti-Ly49G2 (clone 4D11), anti-CD226 (clone 10E5), anti-CCR7 (clone 4B12), anti-Tbet (clone 4B10), anti-Eomes (clone Dan11mag), anti-Granzyme A (clone 3G8.5), anti-Ki67 (clone SolA15), anti-KLRG1 (clone 2F1), anti-STAT4 (clone 38/p-Stat4), anti-HA (clone 6 E2).

Cell sorting

Murine NK cells were isolated from the spleen by magnetic cell sorting incubating for 20 minutes at 4°C with a cocktail of biotin-conjugated mAb: anti-CD3ε, CD5 (53-7.3), CD19, Ly6G, F4/80, CD24, CD4, CD8, CD14 and Ter-119. The Anti-Biotin MicroBeads (20 min) were applied in addition with the DEplete program on the autoMACS® Separator (Miltenyi, Biotec

Inc., CA, USA). A total of 50-90% pure NK cells were obtained using this procedure. Cells were then subsequently sorted into different subsets using a FACSAria Cell Sorter (Becton-Dickinson, San Jose, USA). Purity of sorted cell populations was over 98% as checked by flow cytometry.

Immunostaining for Confocal Analysis

Freshly sorted NK cells were seeded in a 96-Well Optical-Bottom Plate pre-coated with 100µg/mL poly-L-lysine for 1h incubation at 37°C. After 4% PFA fixation for 15 min and three PBS washes, blocking step (PBS 3% BSA) for 30min at RT was followed. Immunostainings were performed after a permeabilization step with 0.05% Triton X-100 for 7min. Primary antibody antibodies were diluted in 3% BSA-PBS and added to the cell for one-hour incubation at room temperature. Primary antibodies used in this study include rat anti-CD122 (Bio X Cell, 4 µg/mL); rabbit anti-Eomes (Abcam, 4 µg/mL); FITC-conjugated mouse anti-T-bet (Biolegend, 1/50). After three washes with PBS, cells were incubated with the appropriate Alexa 555 conjugated anti-rat, AF647 conjugated anti-rabbit secondary antibody at 2 µg/mL in 3% BSA-PBS and add to the cells along with DAPI (4 µg/mL) for one-hour incubation at room temperature. After three gentle washes with PBS, cells were observed with a Zeiss LSM 800 laser scanning confocal microscope. The images and relative quantification were processed using Image J software.

Imaging combined with flow cytometry analysis

Sorted NK cells were prepared as described and stained with appropriate antibodies as described in “Flow cytometry”. The well-stained cells were then analyzed by Image Stream X technology (Amnis) at magnification x40 using IDEAS software. Mean fluorescence intensity (MFI) of T-bet and Eomes were analysed by applying masks (IDEAS software) to discriminate nuclear and cytoplasmic area based on the DAPI staining.

Measurement of *in vivo* cell proliferation

Mice were given two continuous intraperitoneal injection of 0.5 mg EdU (BD Bioscience). 12 hours after the last EdU injection, mice were killed and organs harvested. Cells derived from BM and spleen were stained with mAb as described in “Flow cytometry”. After fixation and permeabilization, cells were stained with FITC anti-EdU antibody, according to manufacturer

instructions. EdU incorporation for different cell populations was measured by flow cytometry.

Cell culture and stimulation with cytokines

T-bet and Eomes expression upon cytokine stimulation

2×10^6 splenocytes from C57BL/6 mice were prepared and then were cultured for 24 h with/without following cytokines IL-12 (25 ng/ml), IL-18 (5ng/ml) and IL-15 (100ng/ml). Cells were collected and were stained with anti-NK1.1, anti-CD3, followed by intracellular staining with anti-T-bet and anti-Eomes before analysis by flow cytometry.

IFN- γ production and degranulation upon cytokine stimulation

1.5×10^6 splenocytes were cultured with Golgi-stop (BD Biosciences) in the presence of anti-CD107a for 4 h. Cytokines were used at the following concentrations: IL-12 (25 ng/ml), IL-18 (5ng/ml). Surface and intracellular stainings were then performed and IFN- γ production as well as CD107a exposure was measured by flow cytometry.

Adoptive transfer

NK cells were sorted into two populations CD11b- (CD11b-CD27+) and DP (CD11b+CD27+) as previously described. Purity of sorted cell populations was more than 98% as checked by flow cytometry. For cell transfer, 2×10^4 to 4×10^5 sorted cells were injected intravenously into unirradiated Ly5a X B6 (CD45.1/2) mice. The presence of transferred cells was analyzed at days 14 after transfer by cytofluorimetric analysis of NK cell-enriched splenocytes. The expression of CD27 and CD11b was analyzed on CD19- CD3- NK1.1+ CD49a-CD49b+ cells.

Cell cytotoxicity assay

Sorted NK cell subsets were plated in 96-well, V-bottom plates and co-cultured for 4h with RMA-KR target cells (MHC I deficient and Rae1b positive) expressing the Nanoluciferase (lentivirus-mediated expression) at a concentration of 100 cells/well. Different ratio of NK to target cells were used: 30:1, 10:1, 3:1, 1:1, 0:1. After NK cell killing RMA-KR-derived NanoLuc was released in the culture supernatant. NanoLuc activity in the culture supernatant thus reflects target cell lysis. The total volume in culture wells was 200 μ L, and plates were centrifuged briefly for 4 min at 2000 rpm. 50 μ L of culture supernatants was collected, and

NanoLuc activity was determined by adding 50 μ L of NanoLuc reagent in black, flat-bottom, 96-well plates. Bioluminescence was measured for 0.1 s with a luminometer (TECAN).

RNA-seq

NK cells were first purified by AutoMACS to get higher purity. And then cells were stained in combination with anti-Cd3, anti-NK1.1, anti-Cd49a, anti-Cd49b, DAPI and subsequently sorted into different subsets (CD11b-, DP, CD27-) using a FACSria Cell Sorter (Becton-Dickinson, San Jose, USA). Purity of sorted cell populations was over 98% as measured by flow cytometry. RNA libraries were prepared as previously described⁶⁴. Briefly, total RNA was purified using the Direct-Zol RNA microprep kit (Zymo Research) according to manufacturer instructions and was quantified using QuantiFluor RNA system (Promega). 1 μ L of 10 μ M Oligo-dT primer and 1 μ L of 10mM dNTPs mix were added to 0.15ng of total RNA in a final volume of 2.3 μ L. Oligo-dT were hybridized 3min at 72°C and reverse transcription (11 cycles) was performed. PCR pre-amplification was then conducted using 16 cycles. cDNA were purified on AmpureXP beads (Beckman Coulter) and cDNA quality was checked on D5000 screen tape and analysed on Tape station 4200 (Agilent). 3ng of cDNA were tagged using Nextera XT DNA sample preparation kit (Illumina). Tagged fragments were further amplified and purified on AmpureXP beads (Beckman Coulter). Tagged library quality was checked on D1000 screen tape and analyzed on Tape station 4200 (Agilent). Sequencing was performed by the GenomEast platform, a member of the “France Génomique” consortium (ANR-10-INBS-0009), on an Illumina HiSeq 4000 sequencing machine (read length 1x50nt).

Generation of Eomes-HAV5/Knockin mice

A homologous recombination plasmid was designed to target mouse Eomes gene. A targeting vector containing Homology sequences to the Eomes locus and an ires-GFP-loxP-tACE-Cre-PGK-gb2-neo-loxP cassette was made previously⁴. Using Red/ET cloning (Gene Bridges), we replaced the IRES-GFP and upstream STOP codon by a 2xHAV5 tag (Hemagglutinin and Parainfluenza virus 5 V/P tag) and terminal STOP codon. The rest of the cassette allows selection with neomycin in both bacteria and eukaryotic cells and is auto-excisable in male mice thanks to Cre expression under the control of the testis-specific Tace promoter and the loxP sites. JM8.A3 ES cells (C57BL/6N) were transfected with this construct

and G418 - resistant clones were obtained. We checked for correct homologous recombination by PCR followed by southern blot using different probes. Chimeric mice were obtained following microinjection of ES cells into C57BL/6 blastocysts and germline transmission was monitored by PCR using different sets of primers encompassing different parts of the targeted locus. The following primer sequences were used for genotyping the animals: Ex6 - F (ACTACCATGGACATCCAGAATGAGC) and Ex6 - R (CAAAGAACACAACAAAACACCACCA). The knock - in fragment size is 520bp and the WT fragment size is 359bp.

Generation of Tbet-HAV5/Knockin mice

A homologous recombination plasmid was designed to target the mouse Tbet gene. Using Red/ET cloning (Gene Bridges), we first replaced Tbet stop codon in a bacterial artificial chromosome containing Tbet genomic sequence (clone number RP24-161-F21; CHORI) by a 2xHAV5-STOP-loxP-tACE-Cre-PGK-gb2-neo-loxP cassette. We then retrieved the cassette with flanking Tbet genomic sequence spanning 3.5kb upstream and 3kb downstream of original stop codon. Standard procedure was used to generate genetically modified JMA8.A3 ES cells and mice (refer to Eomes-HAV5 KI mice procedure). The following primer sequences were used for genotyping the animals: Ex6 - F (GCGAAGGAGACACTAAGAGGAGGAG) and Ex6 - R (AGCACCAGGTTCGTGACTGTAGTTC). The knock - in fragment size is 509bp and the WT fragment size is 356bp.

ChIP-seq

For sample preparation, 10 million NK cells from pooled spleens of HAV5-tagged T-bet and Eomes mice were isolated and crosslinked with 1% formaldehyde for 10 min at 37°C. Crosslinking was stopped with 0.125 M glycine on a roller for 5min. Cells were then washed twice with ice-cold Wash Buffer including detergent (Active motif, 53042). Flash freeze the cell pellet by immersing tubes into dry ice for 10 min. Resuspend each pellet in 5ml ice-cold Swelling Buffer (Active motif, 53042) supplemented with protease inhibitor cocktail (PIC) and PMSF for 30 min on ice. Pellets were then suspended in 300ul SDS buffer (0.5% SDS, 10mM EDTA, 50mM Tris-HCl pH8) supplemented with PIC and PMSF and incubated on ice for 10min. Chromatin were then sheared using a Probe Sonicator device (Active motif) to obtain a

fragments size range between 200 and 1000 bp. After clearance by centrifugation at 4°C, sheared chromatin was used for immunoprecipitation of HA (4µg, CST, catalog number 3724), or normal rabbit IgG control (4 µg, Diagenode, catalog number C15410206) incubated overnight at 4 °C. Protein G magnetic-activated beads (Active Motif, catalog number 53034) were added to each immunoprecipitation reaction and incubate for 3h at 4 °C. Each IP were washed five times with Wash buffer (Active motif, 53042) and chromatin-antibody complexes were eluted with Elution Buffer AM4. Chromatin was then reverse cross-linked and DNA was purified according to the manufacturer's instructions (Active motif). Before sequencing, we pooled three ChIPs for the same mice. Paired-end sequencing was performed on HiSeq 4000 (Illumina) at the GenomEast platform.

Bio-informatic analysis (RNA-seq, ChIP-seq)

RNA-seq: Reads were processed using an in-house RNA-seq pipeline of GenomEast facility. Briefly: raw data were preprocessed using cutadapt 1.10⁶⁵ in order to remove adaptor and low-quality sequences (Phred quality score below 20). Reads shorter than 40 bp were removed for further analysis. Remaining reads were mapped to mouse rRNA sequences using bowtie 2.2.8⁶⁶ and reads mapped to rRNA sequences were discarded for further analysis. Remaining reads were aligned to mm10 assembly of the mouse genome with STAR 2.5.3a⁶⁷. Gene quantification was performed with htseq-count 0.6.1p1⁶⁸ using "union" mode and Ensembl 96 annotations. Differential gene expression analysis between groups of samples was performed using method implemented in the Bioconductor R package DESeq2 1.16.1⁶⁹, with the following non-default options: betaPrior=TRUE, alpha=0.05. P-values were adjusted for multiple testing using the Benjamini and Hochberg method.

ChIP-seq; Fastq files were controlled for %duplication and %GC. Fastq files were aligned to MM10 genome using the Bowtie tool on R software. Peak-calling was done with MACS2. Peak-calling was purified by IDR analysis (100 times peak-calling). Peaks were annotated by the nearest gene method using the ChIPseeker tool on R software. ChIP-seq datas were used for consensus motif research using the Peak-motifs tool on RSAT software with 1 to 2 mismatches allowed in substitution parameter⁴⁸. TSS distance calculus and clustering were obtained with motif-clustering tool on RSAT software. Consensus motifs obtained for each

cluster were associated with a list of transcription factors expressed in NK cells, as evaluated using the Immgen Database⁷⁰.

The used software version is R version 3.6.1 (2019-07-05) -- "Action of the Toes" Copyright (C) 2019 The R Foundation for Statistical Computing. Platform: x86_64-w64-mingw32/x64 (64-bit). Used libraries are « gplots », « ggplot2 », « pheatmap », « RColorBrewer »

Statistical analysis

Statistical analyses were performed using Prism 5 (Graph-Pad Software). Two tailed unpaired t-test, paired t-test, and ANOVA tests with Bonferroni correction were used as indicated in the figure legends.

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Author contributions

JZ, AB, MW, DL, DEC, ALM, AR, GD and AM performed experiments. RS performed in silico analyses. JC, LG, YGH, LB provided reagents, conceptual insight and helped writing the paper. TW wrote the paper and supervised the work.

Conflict of interest

The authors declare no conflict of interest

Figure 1

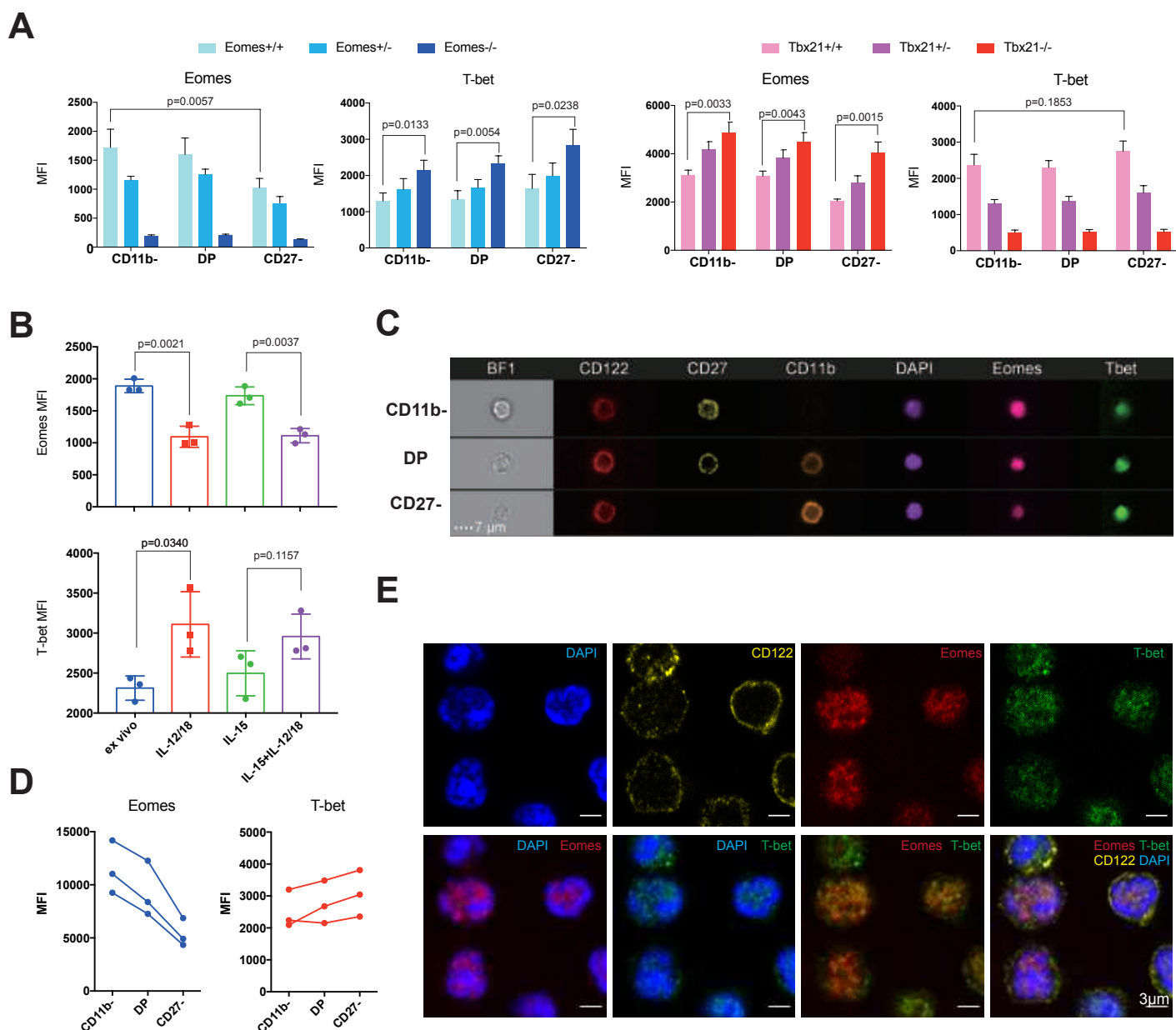


Figure 1. Eomes and T-bet balance each other during NK cell maturation. (A) Flow cytometry measurement of T-bet and Eomes in gated NK cells from WT (*Tbx21*^{+/+}), *Tbx21*^{+/-}, *Tbx21*^{-/-}, *Ncr1*^{Cre/+} (*Eomes*^{+/+}), *Ncr1*^{Cre/+} X *Eomes*^{lox/+} (*Eomes*^{+/-}) and *Ncr1*^{Cre/+} X *Eomes*^{lox/lox} (*Eomes*^{-/-}) mice, as indicated. Bar graphs show the mean \pm SD fluorescence intensity (MFI) of Eomes and T-bet staining in gated NK cell subsets from spleen. Data are representative of 3 experiments and 3 mice are shown for each group. **(B)** WT spleen cells were stimulated O/N in the indicated conditions and T-bet and Eomes expression were measured by flow cytometry. Data are representative of 2 experiments and 3 mice are shown for each group. **(C)** Image stream X (ISX) analysis of T-bet and Eomes expression in gated NK cells. Representative images are shown for the three NK cell subsets. CD122 staining was used to visualize the membrane. **(D)** Quantification of nuclear T-bet and Eomes in gated NK cell subsets analyzed with IDEAS software. Dots correspond to nuclear MFI of T-bet and Eomes for the indicated subsets. N=3. **(E)** Immature NK cells (CD11b⁻) were sorted from spleen of WT mice and subsequently stained for nucleus (DAPI), T-bet, Eomes and CD122 for confocal microscopy analysis. Shown are representative images of each staining and combinations for immature NK cells. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 2

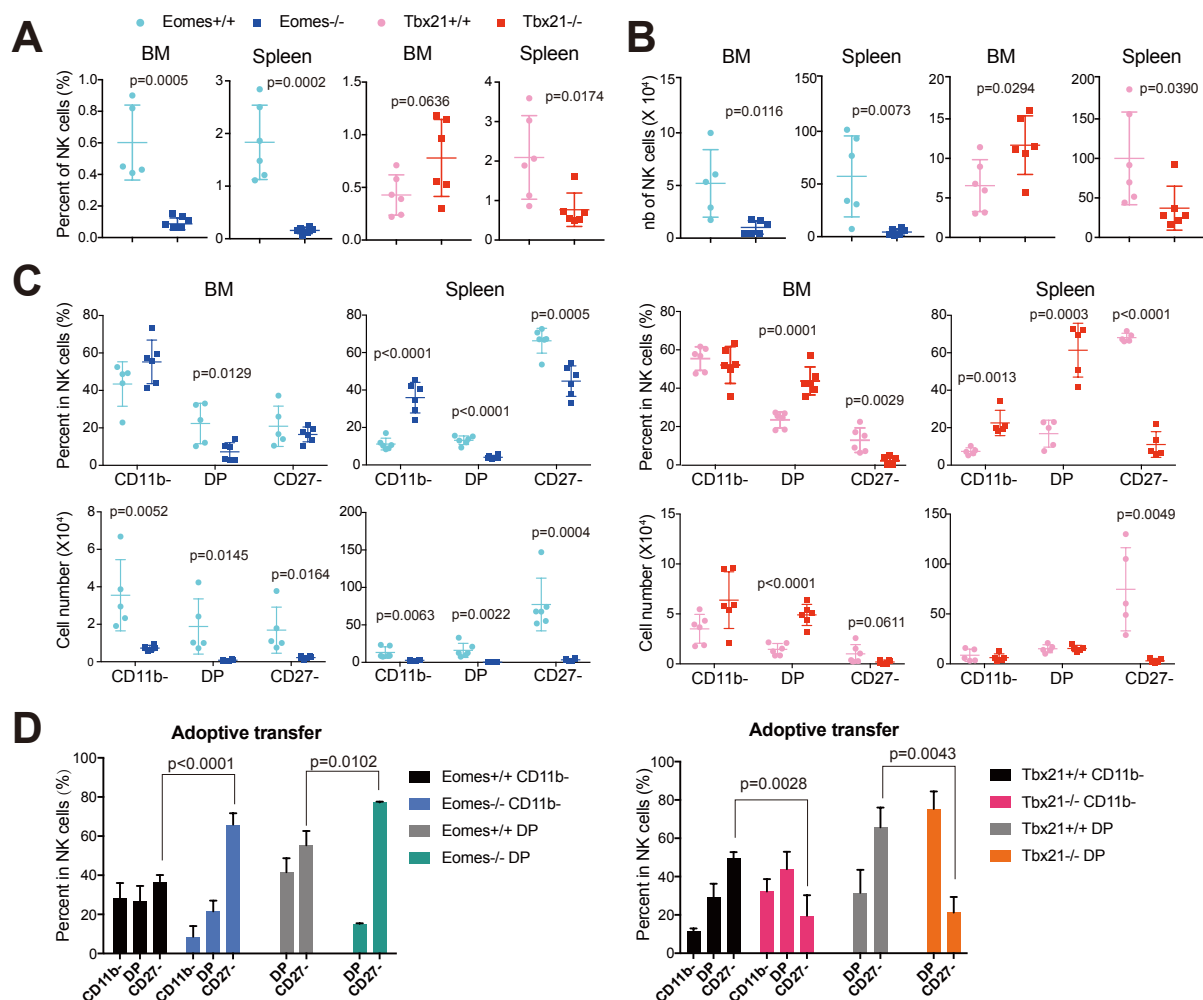


Figure 2. Eomes and T-bet promote NK cell survival and differentiation at different cellular transitions. (A) Flow cytometry analysis of NK cell percentage in BM or spleen lymphocytes of the indicated mice. Each dot corresponds to a single mouse (n=5-6, pooled from 2 experiments), and graphs show the mean percentage +/-SD. (B) Number of NK cells calculated from data in (A) combined with numeration of corresponding organs. (C) Proportion and numbers of NK cell subsets for the mice and organs analyzed in (A-B). (D) NK cells of the indicated subsets (CD11b⁻ or DP) from indicated genotypes were FACS-sorted and then adoptively transferred into congenic unirradiated Ly5a X B6 mice (CD45.1/2). Two weeks later, spleen NK cells were purified from these mice and the CD11b/CD27 phenotype of transferred NK cells (identified by their CD45 expression) was analyzed by flow cytometry. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 3

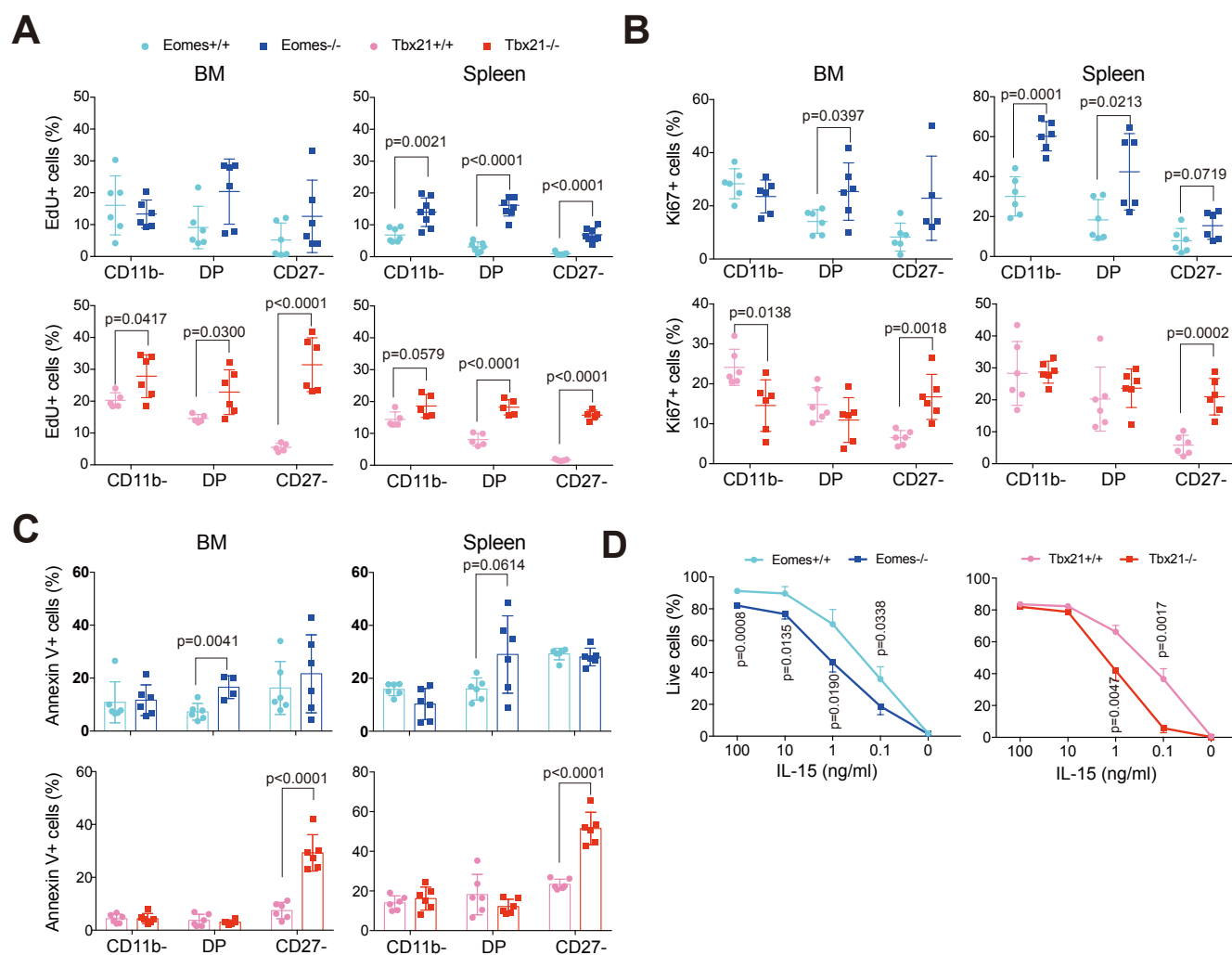


Figure 3. Eomes and T-bet regulate NK cell proliferation and survival at different stages. (A) Mice were injected twice with EdU within a two-day-period, and EdU incorporation was measured in NK cells from the indicated organs by flow cytometry one day after the last injection. Each dot corresponds to a single mouse ($n=6$, pooled from 2 experiments), and graphs show the mean percentage \pm SD. (B) Flow cytometry analysis of Ki67 expression in BM and spleen NK cells. Each dot corresponds to a single mouse ($n=6$, pooled from 2 experiments), and graphs show the mean percentage \pm SD. (C) *Ex vivo* apoptosis of BM and spleen NK cell subsets as assessed by flow cytometry analysis of Annexin-V staining. Bar graphs show the mean percentage \pm SD of Annexin-V⁺ cells within each subset, dots correspond to individual mice analyzed ($n=6$ representative of three experiments). (D) Total NK cell viability (% Annexin-V negative cells) after culture of splenocytes with IL-15 for 48h at the indicated concentrations. Data are from 3 mice representative of 2 independent experiments. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 4

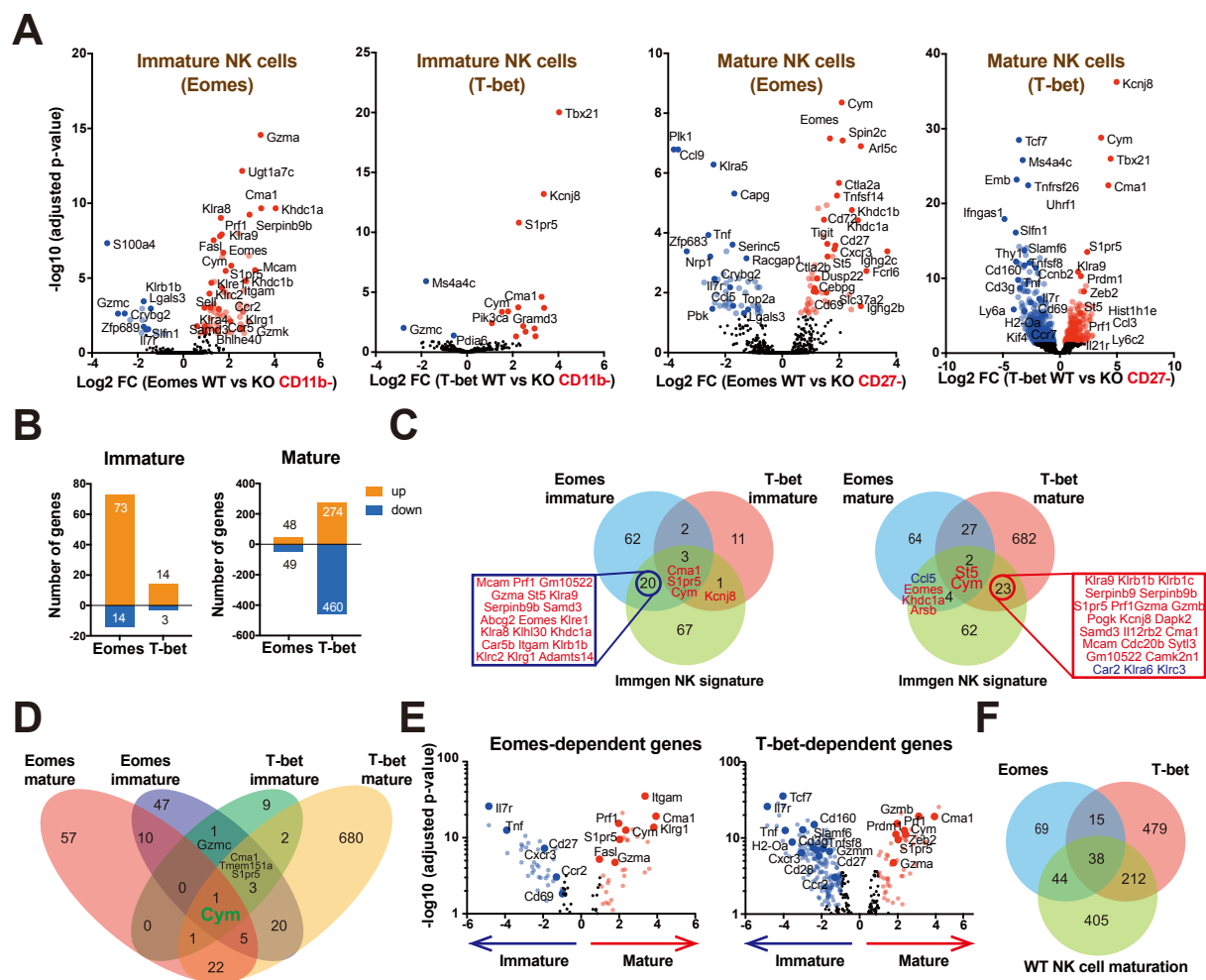


Figure 4. Eomes and T-bet have mostly distinct roles on NK cell transcription during maturation. RNA-seq analysis of sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control *CD11b*⁻ and *CD27*⁻ NK cells (n=3 per group with three sorts in total). DEGs were selected based on adjusted p-value<0.05. **(A)** Volcano plots of gene expression in immature *CD11b*⁻ or mature *CD27*⁻ NK cells comparing T-bet or Eomes deficient cells with appropriate controls. Selected genes are highlighted with their names. **(B)** Bar histograms showing the number of genes induced or repressed by T-bet or Eomes in immature and mature NK cells. **(C)** Venn diagrams showing the overlap between the Immgen-defined NK cell signature and the Eomes-activated and T-bet-activated genes in immature and mature NK cells. **(D)** Venn diagram showing the overlap between T-bet regulated and Eomes-regulated genes in immature and mature NK cells. **(E)** Volcano plots comparing the expression of T-bet or Eomes dependent genes between WT immature and mature NK cells. Selected genes are highlighted with their names. **(F)** Venn diagram showing the overlap between T-bet-dependent, Eomes-dependent and maturation-regulated genes.

Figure 5

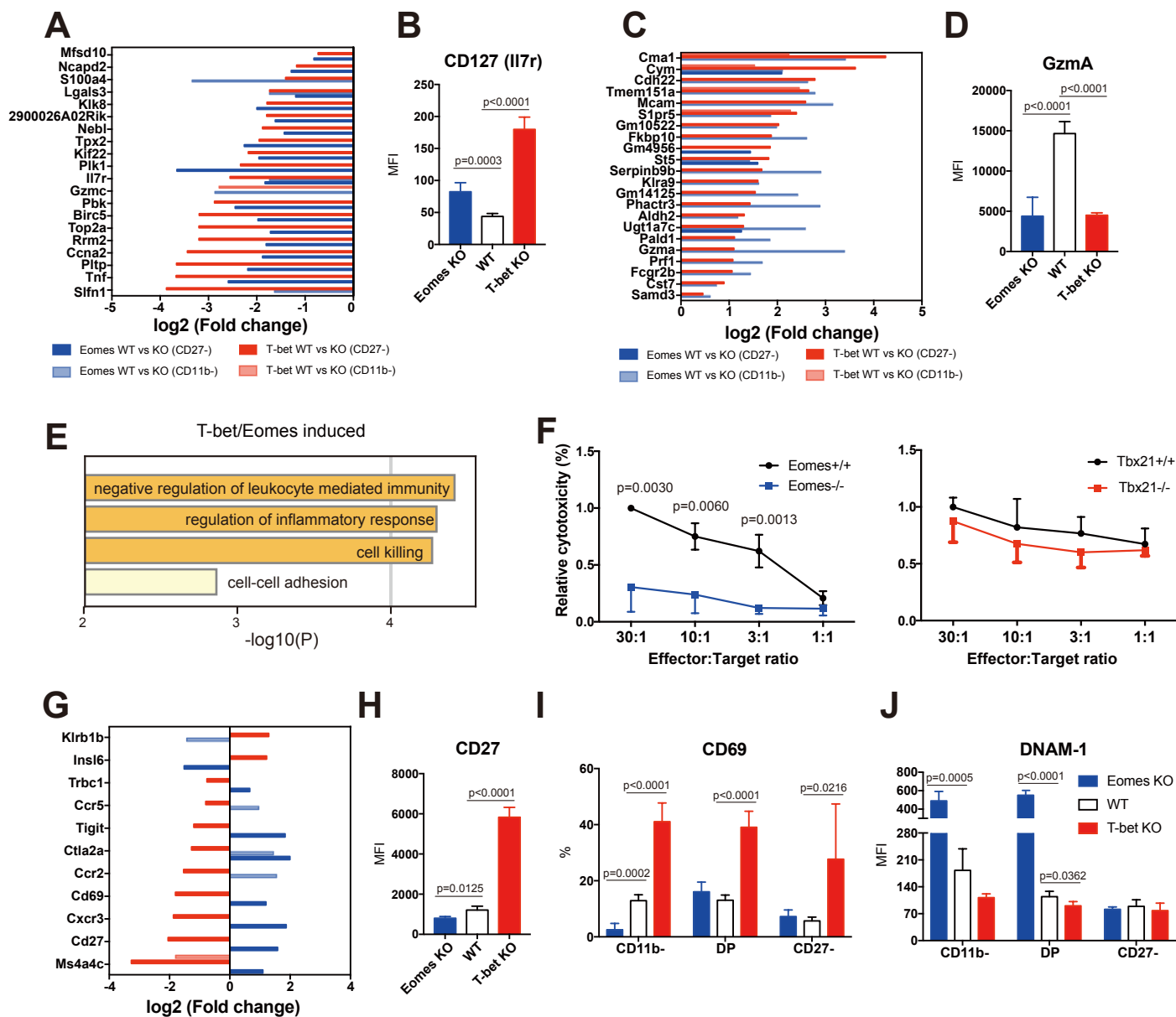


Figure 5. Shared transcriptional activities of T-bet and Eomes in NK cells. RNA-seq analysis of sorted Tbx21^{-/-}, Eomes^{-/-} and appropriate control CD11b⁻ and CD27⁻ NK cells (n=3 per group with three sorts in total). DEGs were selected based on adjusted p-value < 0.05. **(A)** T-bet/Eomes co-repressed genes in immature or mature NK cells. Bar graph showing the log₂ transformed fold change of genes between controls and T-bet or Eomes deficient mice, as indicated. **(B)** Bar graph showing the mean \pm SD of IL7R expression in NK cells of the indicated genotype as measured by flow cytometry. The different controls for each genotype are pooled and annotated as “WT” in the graph which is same for the following graphs in (D, H-J). N=3. **(C)** T-bet/Eomes co-induced genes in immature or mature NK cells. Bar graph showing the log₂ transformed fold change of genes between controls and T-bet or Eomes deficient mice, as indicated. **(D)** Bar graph showing the mean \pm SD of GzmA expression in NK cells of the indicated genotype as measured by flow cytometry. N=3. **(E)** Functional annotation of the T-bet/Eomes induced gene set using Metascape. Bar graphs show selected terms among the most significant ones. **(F)** Cytotoxicity assay using sorted Tbx21^{-/-}, Eomes^{-/-} and appropriate control CD27⁻ NK cells as effectors and YAC1-Nano-luc cells as targets. The YAC-1 lysis of control group 30:1 (E:T) was set as 100%. **(G)** Bar graph showing the log₂ transformed fold change of genes regulated in opposite ways by T-bet and Eomes, between controls and T-bet or Eomes deficient mice, as indicated. **(H-J)** Bar graphs showing the mean \pm SD of (H) CD27, (I) CD69 and (J) DNAM-1 expression in NK cells of the indicated genotype as measured by flow cytometry. N=3. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 6

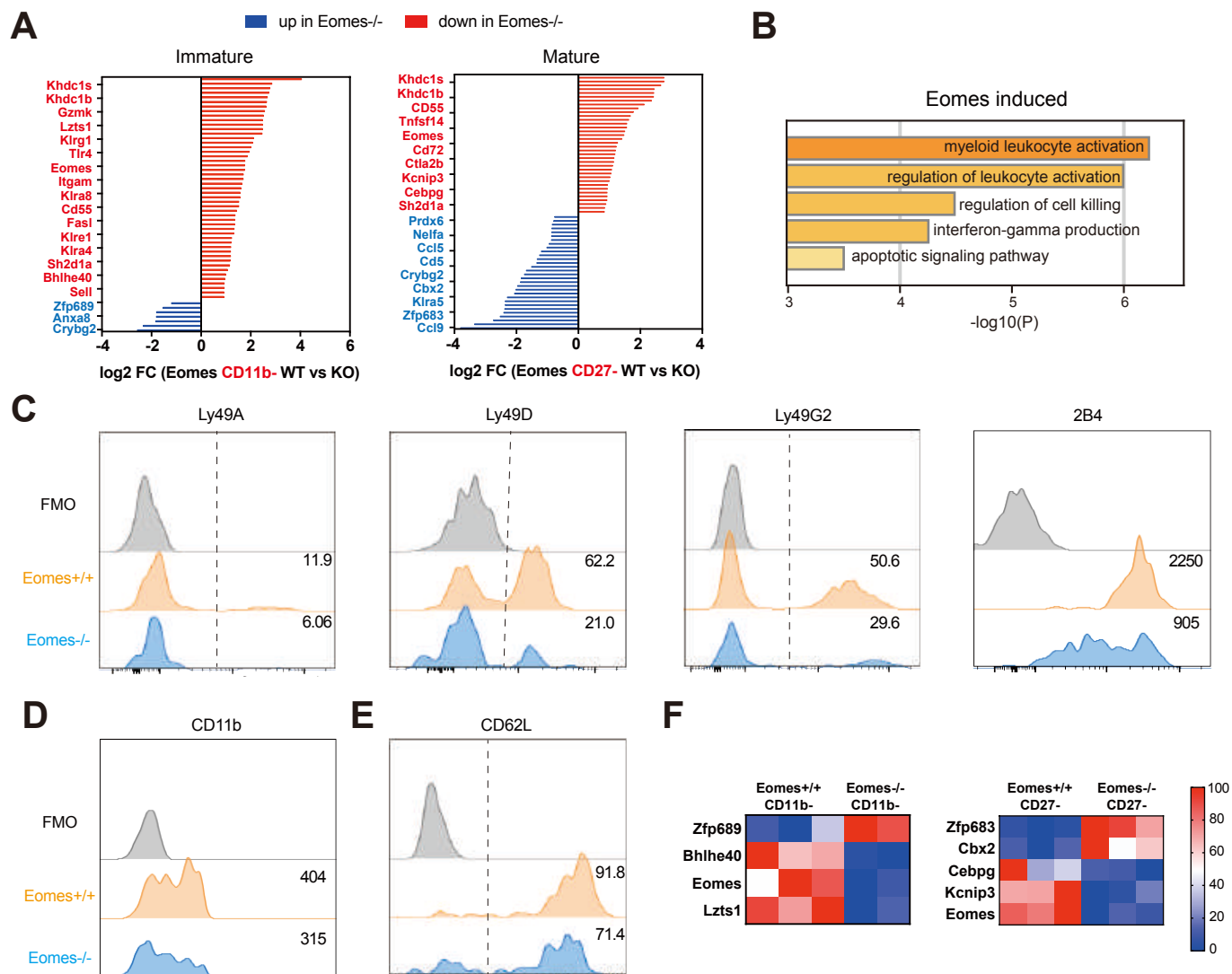


Figure 6. Eomes-specific role in the induction of hallmark NK cell genes. RNA-seq analysis of sorted Tbx21^{-/-}, Eomes^{-/-} and appropriate control CD11b⁻ and CD27⁻ NK cells (N=3 per group with three sorts in total). DEGs were selected based on adjusted p-value<0.05. **(A)** Genes specifically regulated by Eomes in immature or mature NK cells. Bar graph showing the log₂ transformed fold change between controls and Eomes deficient NK cells, as indicated. A few selected gene names are shown. **(B)** Functional annotation of the Eomes induced gene set using Metascape. Bar graph showing selected terms among the most significant ones. **(C-E)** Overlaid FACS histograms showing the expression of the indicated surface molecules in Eomes deficient vs control NK cells, as indicated. Grey histograms show control staining. **(F)** Heatmaps showing the log₂ transformed fold change in the expression of the indicated TFs between control and Eomes-deficient NK cells.

Figure 7

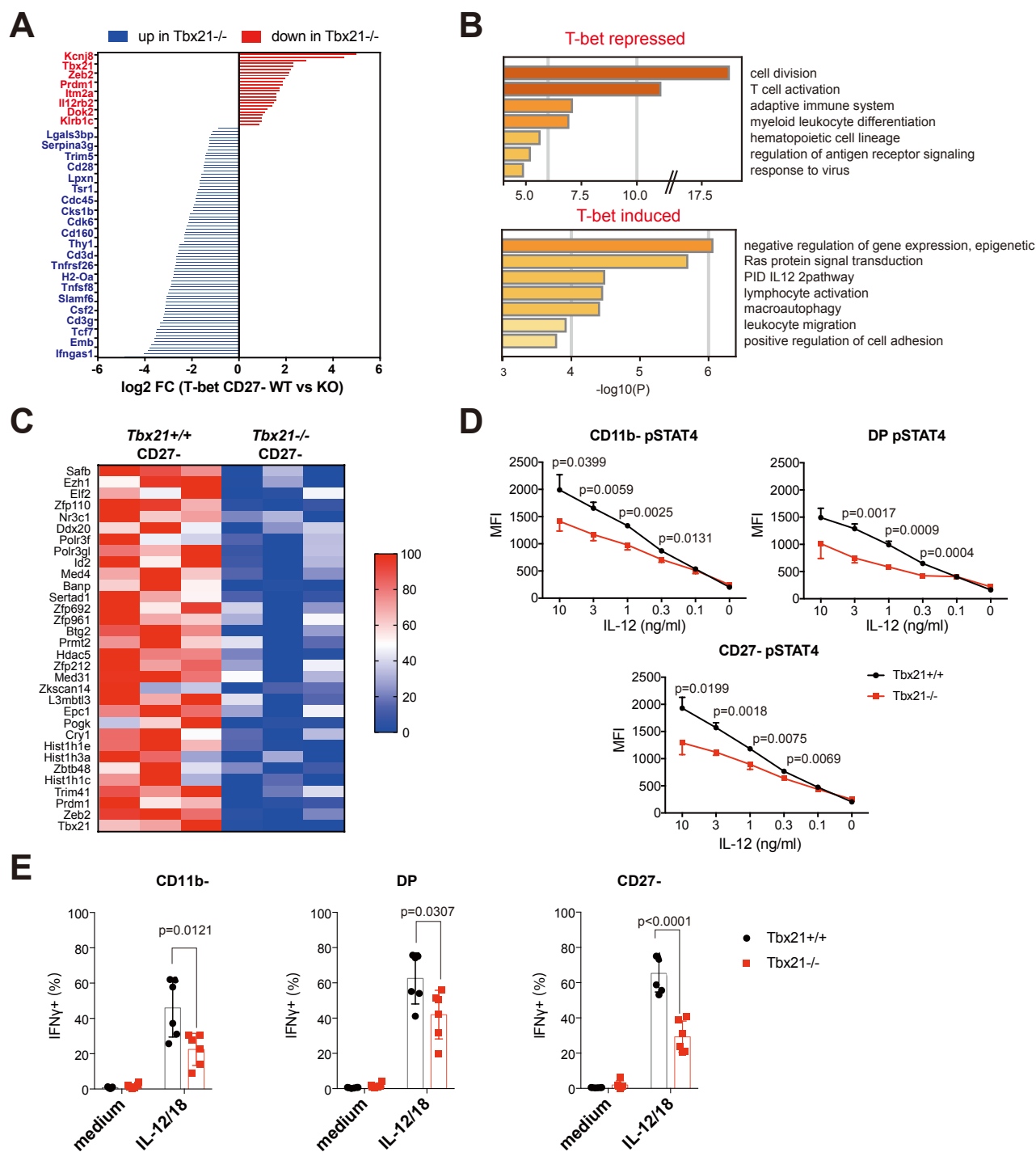


Figure 7. T-bet-specific role in the repression of pluripotency and cell proliferation. RNA-seq analysis of sorted *Tbx21*^{-/-}, *Eomes*^{-/-} and appropriate control CD11b- and CD27- NK cells (n=3 per group with three sorts in total). DEGs were selected based on adjusted p-value < 0.05. **(A)** Genes specifically regulated by T-bet in mature CD27- NK cells. Bar graph shows the log₂ transformed fold change between controls and T-bet deficient mice, as indicated. A few selected gene names are shown. **(B)** Functional annotation of the T-bet-repressed and T-bet-induced genesets using Metascape. Bar graphs show selected terms among the most significant ones. **(C)** Transcription factors, histone subunits or histone modifying enzymes whose expression are dependent on T-bet. The heatmap shows the log₂ transformed fold change in expression between control and T-bet deficient mature NK cells. **(D)** Flow cytometry analysis of STAT4 phosphorylation in WT vs T-bet deficient NK cells of the indicated subsets in response to stimulation with IL-12 for 1h. N=3. **(E)** Flow cytometry analysis of intracellular IFN γ expression in gated spleen NK cells of the indicated genotype following culture in medium supplemented with or without IL-12 and IL-18. N=6 mice pooled from 2 experiments. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure 8

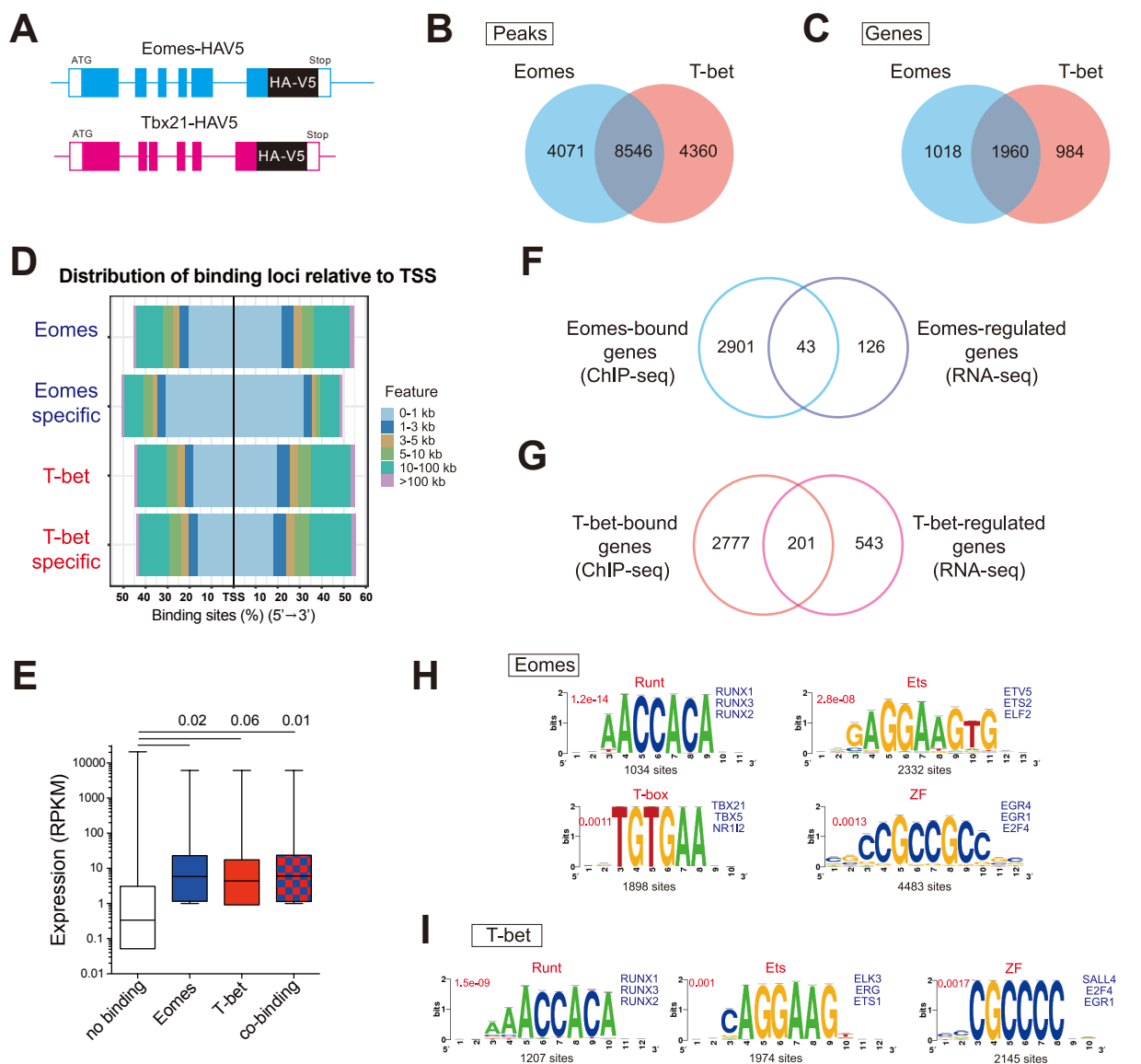


Figure 8. Identification of direct T-bet and Eomes targets using novel KI mice. (A) Scheme of knock-in Eomes and T-bet HAV5 constructs. **(B)** Venn diagram showing the overlap between Eomes and T-bet ChIP-seq peaks. **(C)** Venn diagram showing the overlap between genes near Eomes and T-bet peaks. **(D)** Plot showing the distribution of T-bet and Eomes peaks in the mouse genome based on the distance to TSS. **(E)** Histogram of mean transcript expression with respect to T-bet and/or Eomes binding according to RNA-seq data. **(F)** Venn diagram showing the overlap between Eomes-bound (ChIP-seq) and Eomes-regulated genes (RNA-seq). **(G)** Venn diagram showing the overlap between T-bet-bound (ChIP-seq) and T-bet-regulated genes (RNA-seq). **(H-I)** Motifs associated with (H) Eomes or (I) T-bet peaks and corresponding TFs ranked by significance.

Figure S1 related to Figure 1

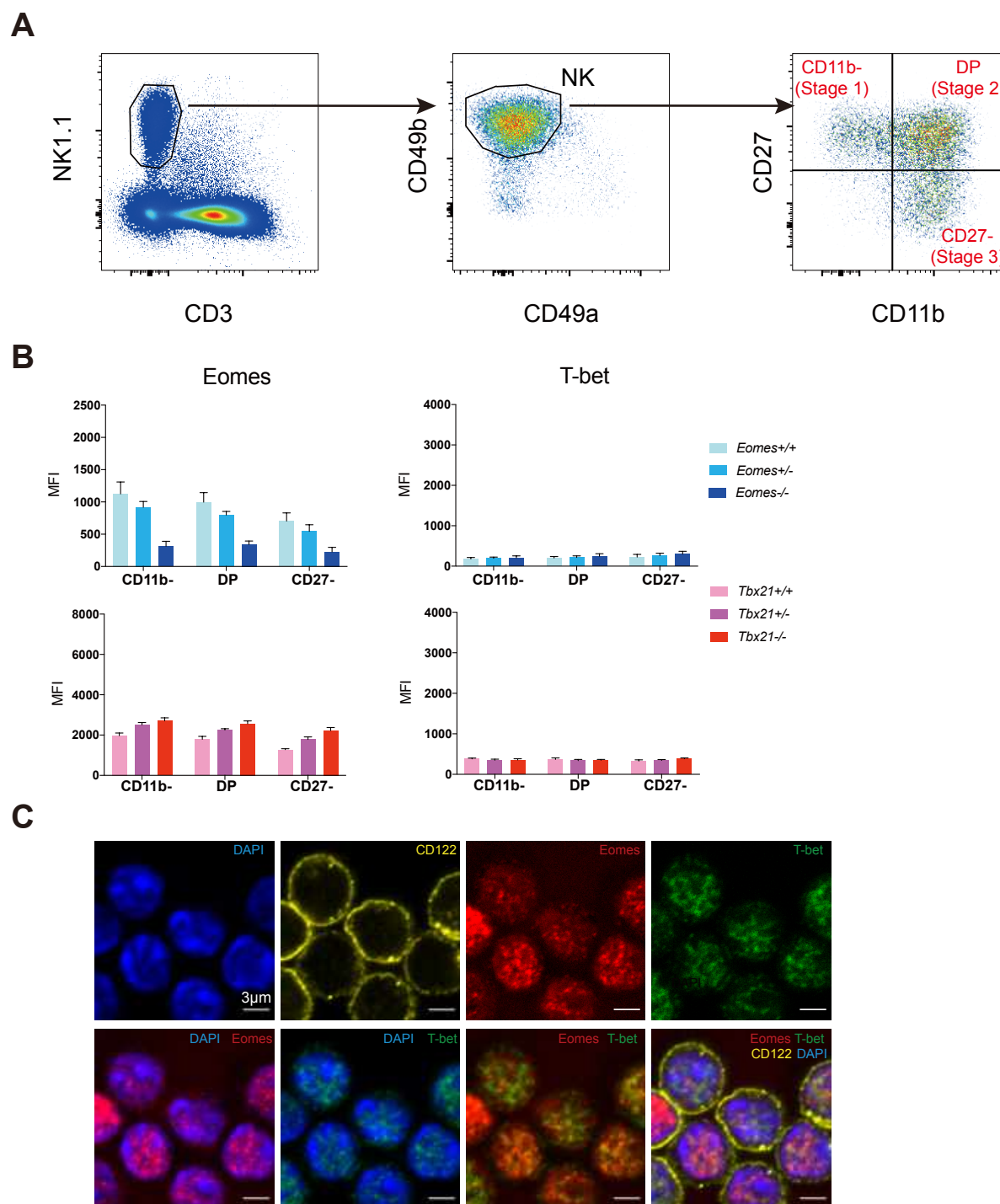


Figure S1. Extended data for Figure 1. (A) Gating strategy of NK cells in this study. NK cells were defined as NK1.1+CD3-CD49b+CD49a-. NK cell subsets CD11b-, DP and CD27- were gated as shown in the right panel. (B) Flow cytometry measurement of T-bet and Eomes in gated NK cells from WT (*Tbx21*^{+/+}), *Tbx21*^{+/-}, *Tbx21*^{-/-}, *Ncr1*^{Cre/+} (*Eomes*^{+/+}), *Ncr1*^{Cre/+} X *Eomes*^{lox/+} (*Eomes*^{+/-}) and *Ncr1*^{Cre/+} X *Eomes*^{lox/lox} (*Eomes*^{-/-}) mice, as indicated. Bar graphs show the mean \pm SD fluorescence intensity (MFI) of Eomes and T-bet staining in gated NK cell subsets from the BM. Data are representative of 3 experiments and 3 mice are shown for each group. (C) Mature NK cells (CD27-) were sorted from WT mice and subsequently stained for nucleus (DAPI), T-bet, Eomes and CD122 for confocal microscopy analysis. Shown are representative images of each staining and combinations for mature NK cells.

Figure S3 related to Figure 4

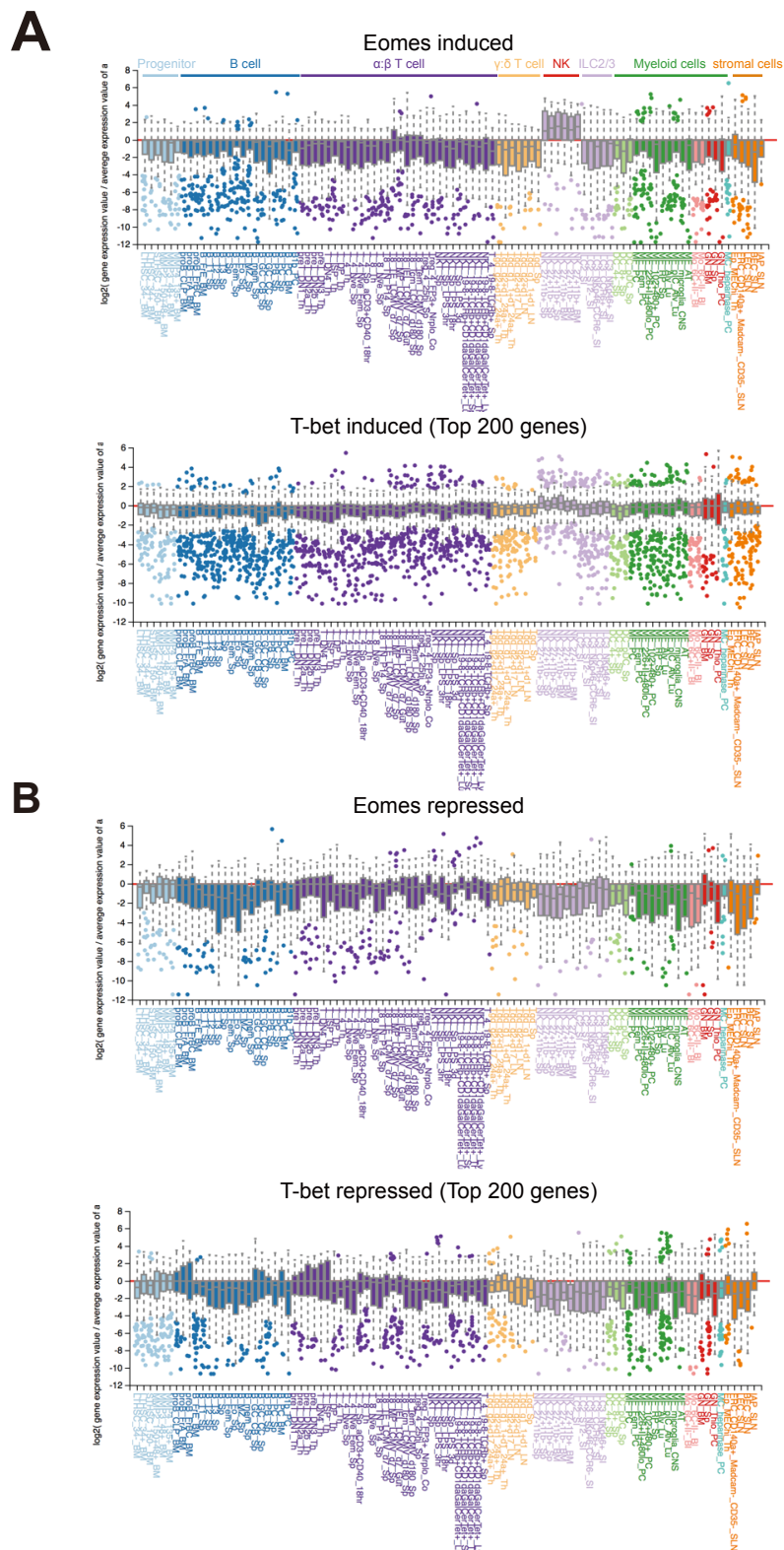


Figure S3. Expression pattern of NK cell genes regulated by T-bet or Eomes across the immune system. (A) Expression profiles of NK cell genes activated by Eomes or T-bet across immune subsets (Immgen data³⁹). **(B)** Expression profiles of NK cell genes repressed by Eomes or T-bet across immune subsets (Immgen data³⁹).

Figure S4 related to Figure 5

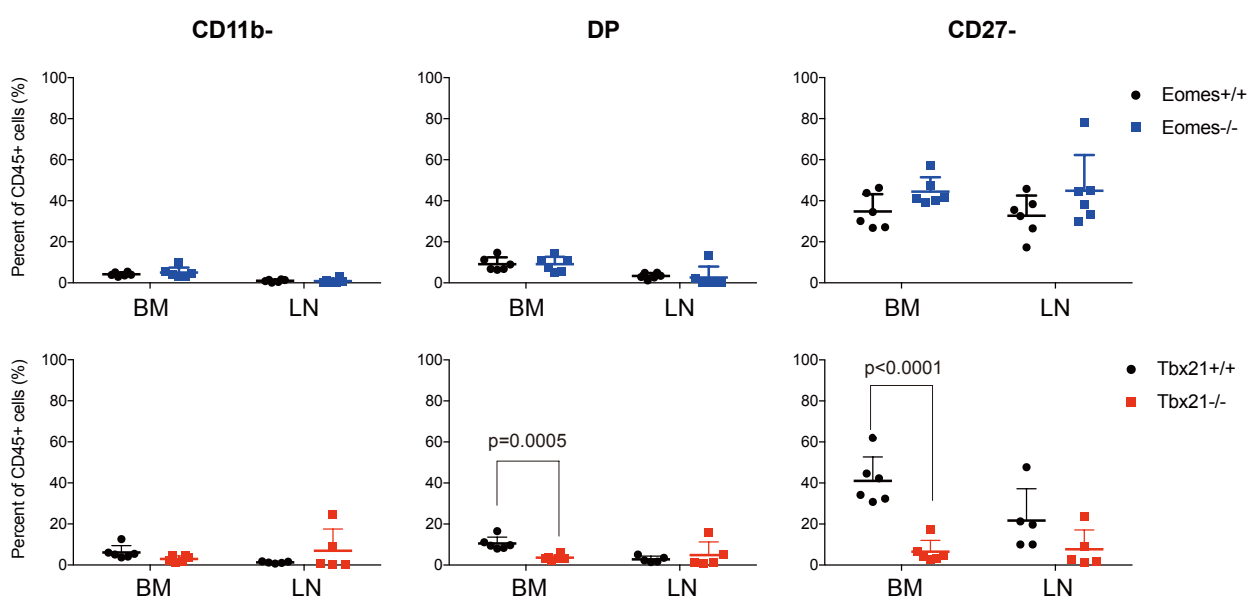
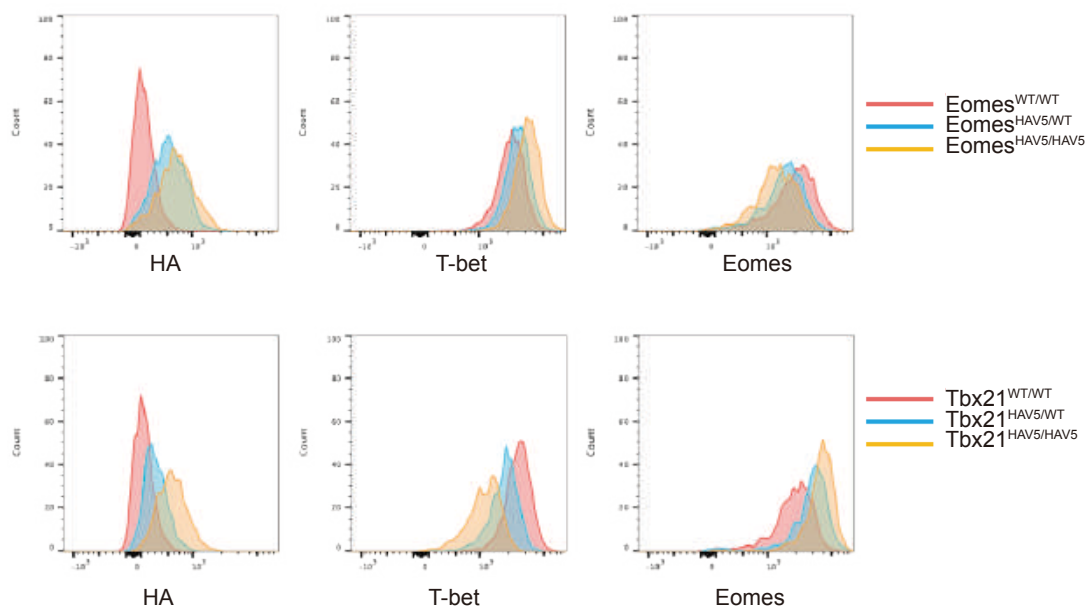


Figure S4. T-bet promotes NK cell blood circulation. Mice of the indicated genotypes were injected with CD45 MAb intravenously 5 minutes before sacrifice. The percentages of CD45+ NK cells among different subsets of different organs were analyzed by flow cytometry. Results were pooled from 2 experiments with N=5-6 mice in total. Paired t-tests were used for statistical analysis of data presented in this figure.

Figure S5 related to Figure 8

A



B

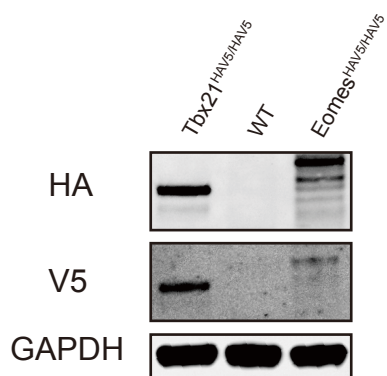


Figure S5. Validation of HA-V5 expression in knock-in mice. (A) Flow cytometry analysis of T-bet, Eomes, and HA expression in gated NK cells from the indicated mouse strains. **(B)** WB analysis of HA and V5 expression in NK cells enriched from the spleen of the indicated mouse strains.

Figure S6

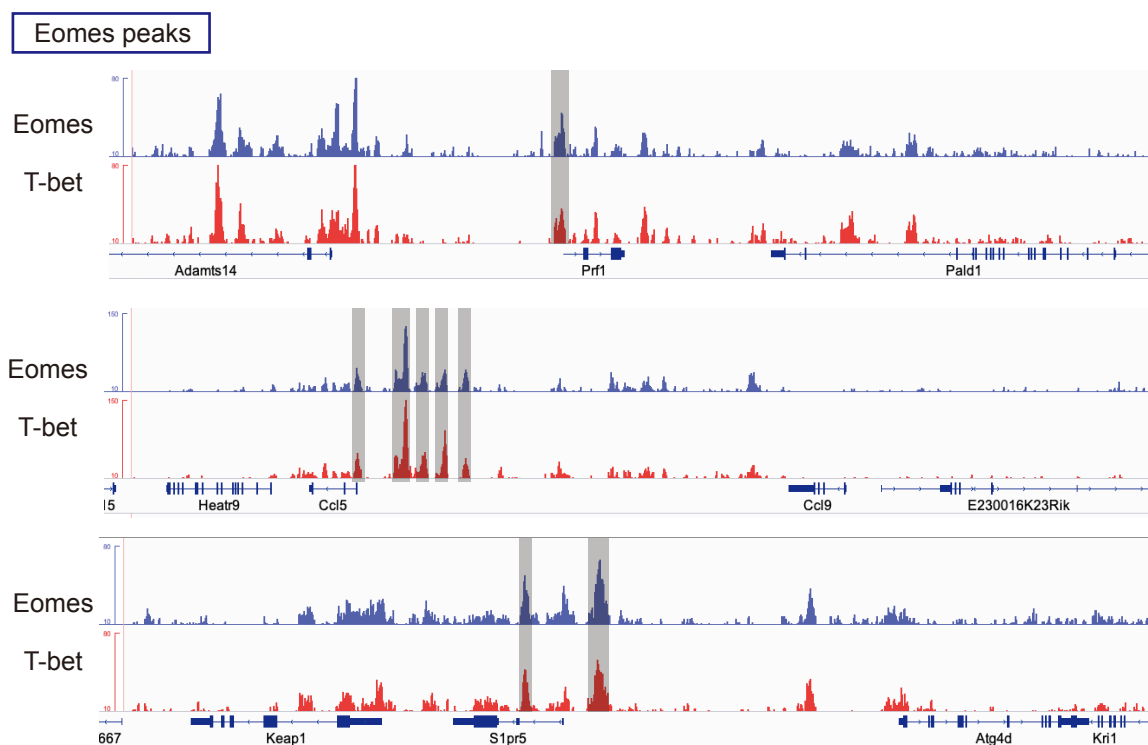


Figure S6. Selected examples of Eomes ChIP-seq tracks for validated functional Eomes targets. Shaded areas correspond to "specific" Eomes peaks.

Figure S7

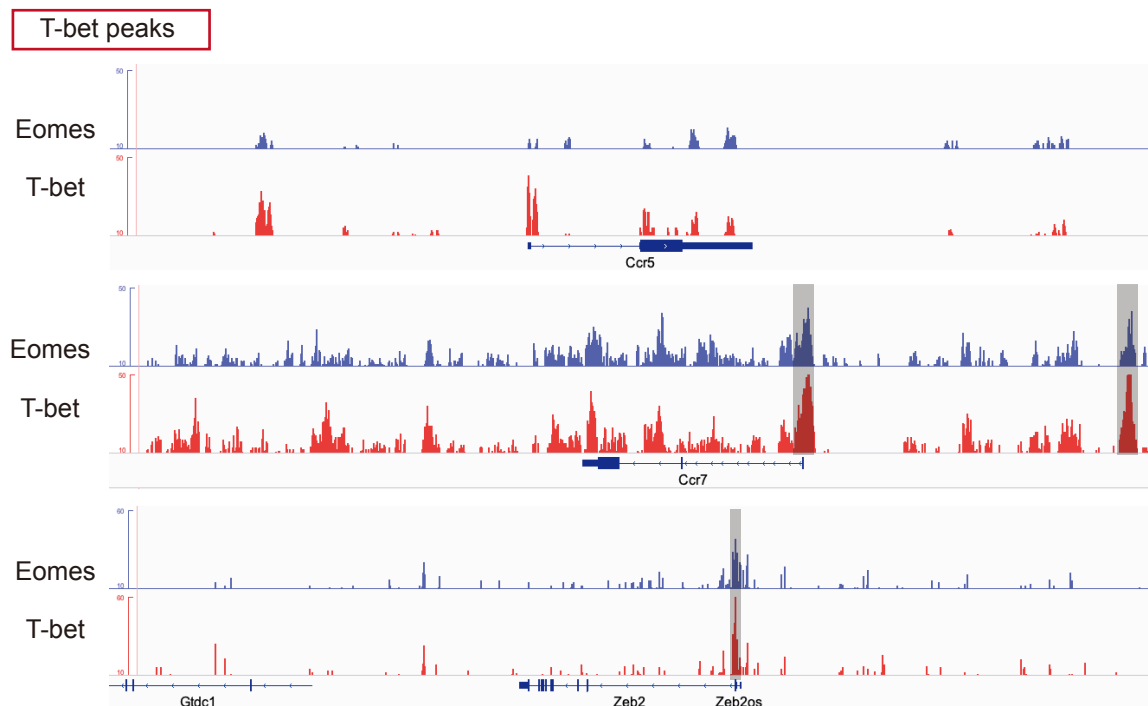


Figure S7. Selected examples of T-bet ChIP-seq tracks for validated functional T-bet targets. Shaded areas correspond to "specific" T-bet peaks.

Figure S8

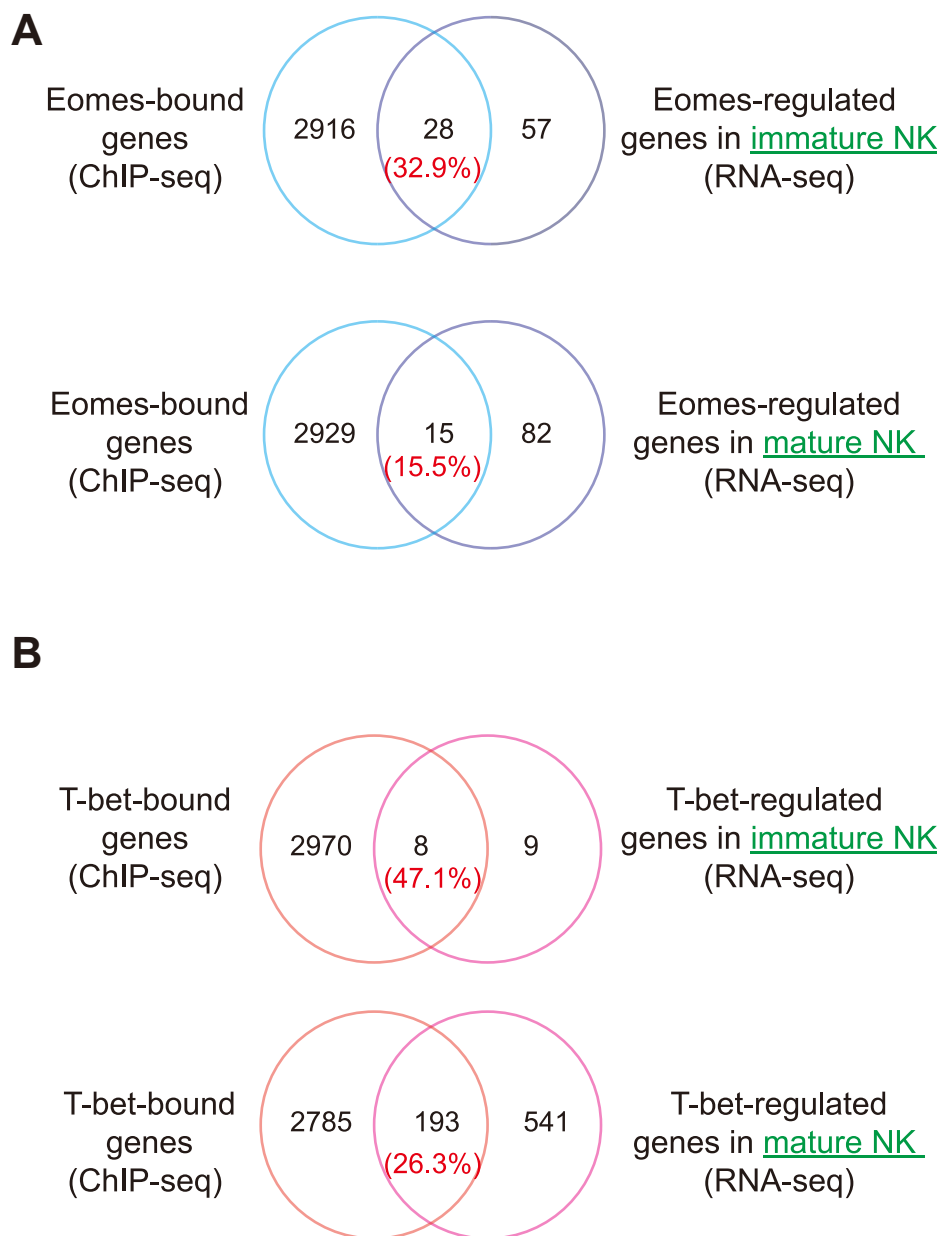


Figure S8. Overlap between ChIP-seq and DEGs in immature and mature NK cells. (A) Venn diagrams showing the overlap between Eomes binding (ChIP-seq) and genes significantly regulated by Eomes in immature or mature NK cells (RNA-seq, p-value<0.05). (B) Venn diagrams showing the overlap between T-bet binding (ChIP-seq) and genes significantly regulated by T-bet in immature or mature NK cells (RNA-seq, p-value<0.05).

Figure S9

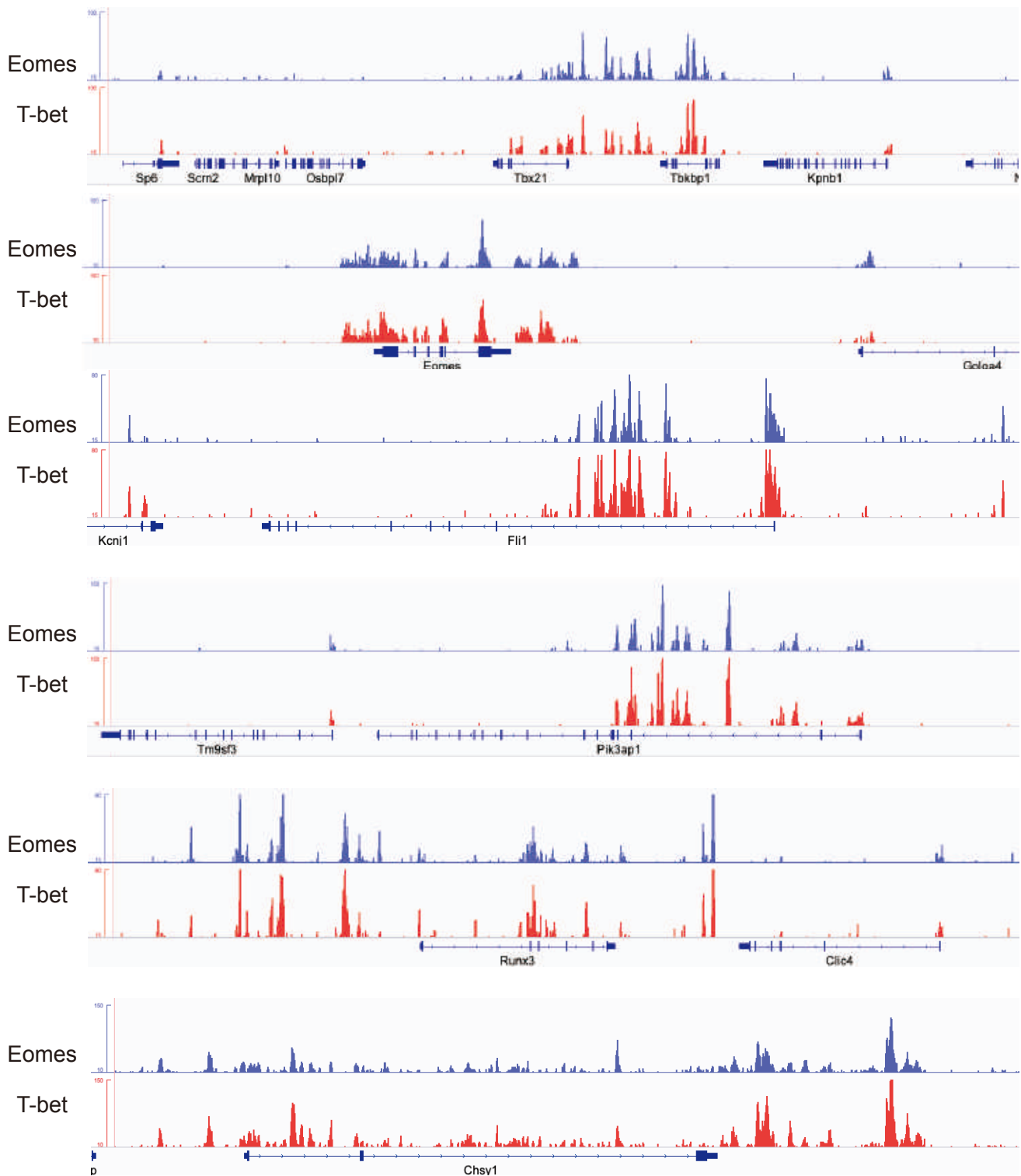


Figure S9. ChIP-seq tracks for genes with a high number of T-bet and Eomes peaks.

4.3 Project 2: Zeb1 in iNKT cell development

4.3.1 Article 2

Zeb1 represses TCR signaling, promotes the proliferation of T cell progenitors and is essential for NK1.1+ T cell development

Jiang Zhang, Mélanie Wencker, Quentin Marliac, Aurore Berton, Uzma Hasan, Raphaël Schneider, Daphné Laubretton, Dylan E. Cherrier, Anne-Laure Mathieu, Amaury Rey, Wenzheng Jiang, Julie Caramel, Laurent Genestier, Antoine Marçais, Jacqueline Marvel, Yad Ghavi-Helm & Thierry Walzer

Cellular & Molecular Immunology (2020)



ARTICLE

Zeb1 represses TCR signaling, promotes the proliferation of T cell progenitors and is essential for NK1.1⁺ T cell development

Jiang Zhang^{1,2}, Mélanie Wencker¹, Quentin Marliac¹, Aurore Berton¹, Uzma Hasan¹, Raphaël Schneider³, Daphné Laubretton¹, Dylan E. Cherrier¹, Anne-Laure Mathieu¹, Amaury Rey¹, Wenzheng Jiang², Julie Caramel⁴, Laurent Genestier⁴, Antoine Marçais¹, Jacqueline Marvel¹, Yad Ghavi-Helm³ and Thierry Walzer¹

T cell development proceeds under the influence of a network of transcription factors (TFs). The precise role of Zeb1, a member of this network, remains unclear. Here, we report that Zeb1 expression is induced early during T cell development in CD4⁻CD8⁻ double-negative (DN) stage 2 (DN2). Zeb1 expression was further increased in the CD4⁺CD8⁺ double-positive (DP) stage before decreasing in more mature T cell subsets. We performed an exhaustive characterization of T cells in *Cellophane* mice that bear *Zeb1* hypomorphic mutations. The *Zeb1* mutation profoundly affected all thymic subsets, especially DN2 and DP cells. Zeb1 promoted the survival and proliferation of both cell populations in a cell-intrinsic manner. In the periphery of *Cellophane* mice, the number of conventional T cells was near normal, but invariant NKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ CD8 T cells were virtually absent. This suggested that Zeb1 regulates the development of unconventional T cell types from DP progenitors. A transcriptomic analysis of WT and *Cellophane* DP cells revealed that Zeb1 regulated the expression of multiple genes involved in the cell cycle and TCR signaling, which possibly occurred in cooperation with Tcf1 and Heb. Indeed, *Cellophane* DP cells displayed stronger signaling than WT DP cells upon TCR engagement in terms of the calcium response, phosphorylation events, and expression of early genes. Thus, Zeb1 is a key regulator of the cell cycle and TCR signaling during thymic T cell development. We propose that thymocyte selection is perturbed in *Zeb1*-mutated mice in a way that does not allow the survival of unconventional T cell subsets.

Keywords: T cell selection; TCR signaling; Zeb1; transcription; development

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INTRODUCTION

T cell development occurs in the thymus and begins in immature thymocytes that are double negative (DN) for CD4 and CD8 expression. The DN population can be subdivided into four subsets, DN1–DN4, depending on the expression of the cell surface molecules CD44 and CD25 (for a review, see¹). DN1 cells (CD44⁺CD25⁻) are the most immature progenitors and retain the ability to differentiate into non-T cell lineages. In DN2 cells (CD44⁺CD25⁺), the expression of RAG1/2 is induced, which promotes the rearrangement of gene segments encoding the TCR- β , TCR- γ , and TCR- δ subunits. In DN3 cells (CD44⁻CD25⁺), the T cell antigen receptor (TCR) β -chain associates with the pre-TCR α -chain and CD3 subunits to form the pre-TCR complex; the pre-TCR complex allows β -selection to occur. During β -selection, DN3 cells with productive TCR β rearrangements receive survival and proliferative signals and mature into the DN4 (CD44⁻CD25⁻) stage. DN4 thymocytes then develop into CD4⁺CD8⁺ double-positive (DP) cells.²

At the DP stage, a series of events takes place that determines the fate of developing T cells, including rearrangement of the TCR alpha locus, association of the $\alpha\beta$ T cell receptor, and subsequent

thymic selection. In general, high-affinity interactions between the $\alpha\beta$ TCR and self-peptide-MHC complexes presented by different thymic cells lead to negative selection and elimination of self-reactive thymocytes, while low-affinity interactions result in positive selection and development of CD4 or CD8 single-positive (SP) T cells.^{3–5} Despite this general rule, regulatory T cells and invariant NKT cells (iNKT) receive stronger TCR signals than conventional T cells during their development⁶ as a result of selection by agonist self-antigens. iNKT cells are a subset of innate-like T cells with a single invariant TCR α chain (Va14-Ja18 in mice) and a limited repertoire of TCR β chains (V β 8.2, V β 7, or V β 2) that recognize glycolipid antigens bound to CD1d, a nonpolymorphic MHC molecule.⁷ iNKT cell development includes discrete stages (stages 0–3) that can be discriminated according to CD44 and NK1.1 expression.⁸ Three functionally distinct iNKT cell subsets have also been identified: iNKT1 cells, which express T-bet and mainly secrete IFN- γ ; iNKT2 cells, which express Gata3 and Plzf and secrete IL-4 and IL-13; and iNKT17 cells, which express Ror γ and secrete IL-17. The TCR signal strength during selection governs the development of iNKT cell subsets, with strong signals promoting iNKT2 and iNKT17 development.^{9,10} A large number of molecules

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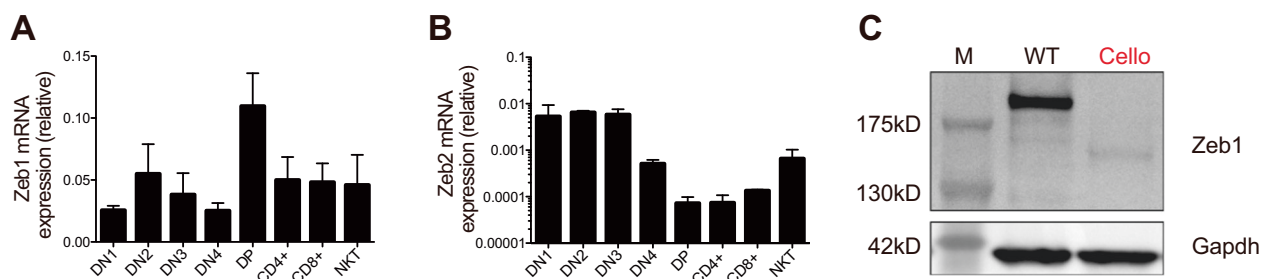


Fig. 1 Zeb1 expression in WT and *Cellophane* thymocytes. **a, b** RT-PCR analysis of RNA from sorted thymocyte subsets isolated from C57BL/6 mice, as indicated. The results are presented relative to the expression of the control gene *Gapdh*. **c** WB analysis of Zeb1 expression in total thymocytes from WT and *Cellophane* mice, as indicated. Data are representative of three independent experiments with three to six mice (**a, b**) or three independent experiments with three mice (**c**)

regulate the strength of TCR-derived signaling. TCR signaling strength can also be modulated at the transcriptional level by transcription factors (TFs) such as Sox4¹¹ or at the posttranscriptional level by miR-181.^{12,13} The loss of either Sox4 or miR-181 blocks iNKT cell development. Mechanistically, miR-181a regulates the expression of multiple phosphatases and other proteins to boost TCR signaling as well as cell metabolism.^{12,13} Interestingly, mice expressing a hypomorphic form of Zap70, a major TCR-proximal kinase, also show impaired developmental maturation of $\gamma\delta$ T cells, suggesting that innate-like T cell subsets are particularly dependent on the tight regulation of the strength of TCR signaling for their development.¹⁴

A dense network of TFs has been shown to regulate T cell development.¹⁵ Early commitment is dependent on Notch signaling,¹⁶ which induces the expression of many TFs and maintains their expression throughout T cell development. Among these TFs, the E-protein family factors E2a, Tcf1 (encoded by *Tcf7*) and Heb (encoded by *Tcf12*)¹⁷ induce the expression of TCR components and balance the survival and proliferation of thymocytes.¹⁸ Many other TFs, such as Gata3, Myb, Runx1, and Bcl11b, also cooperate with E proteins at different developmental stages and further establish T cell identity.^{15,18}

The Zeb family of TFs consists of Zeb1 and Zeb2, which are best known for their role in epithelial-to-mesenchymal transition (EMT). EMT programs operate at different stages of embryonic development and are downstream of Wnt, TGF- β , Bmp, Notch, and other signaling pathways.¹⁹ *Zeb1*^{-/-} mice exhibit multiple developmental defects and die at birth.²⁰ Under pathological conditions, activation of EMT programs contributes to fibrosis and cancer metastasis.²¹ Zeb1 and Zeb2 are highly homologous and are characterized by two clusters of zinc finger domains at the protein extremities. They also contain a homeodomain and a Smad-binding domain and can interact with many other TFs.²² Zeb1 and Zeb2 are also expressed in a tightly regulated manner in the immune system and regulate cell differentiation.²³ We and others have previously shown that Zeb2 regulates terminal NK cell²⁴ and effector CD8 T cell differentiation.^{25,26} Mutated mice expressing a truncated form of Zeb1 without the C-terminal zinc finger clusters at C727 have a small and hypocellular thymus, which is the result of a reduction in early T cell precursors.²⁷ In *Cellophane* mutant mice, a T \rightarrow A mutation in the seventh exon of *Zeb1* replaces the tyrosine at position 902 with a premature stop codon.²⁸ The resulting mRNA encodes a truncated protein lacking the C-terminal zinc finger domain, which is predicted to be hypomorphic. *Cellophane* homozygous mice have small, hypocellular thymi with decreased DP thymocytes. However, the mechanism of Zeb1 action during T cell development and its role in the maturation of T cell subsets remain unclear. Here, we show that *Cellophane* homozygous mice lack several peripheral T cell subsets, including iNKT cells, NK1.1⁺ $\gamma\delta$ T cells, and Ly49-expressing CD8 T cells. This specific defect involving innate-like T cells is caused by the cell-intrinsic role of Zeb1 in T cell

development. We show that Zeb1 expression is maximal in the DN2 and DP stages of T cell development. Furthermore, Zeb1 regulates the transition to the SP stage by promoting cell proliferation and survival and repressing the expression of various molecules that modulate the strength of TCR signaling. Therefore, we propose that Zeb1 is a key regulator of thymocyte selection that is essential for the development and survival of innate-like T cell subsets undergoing agonist-type selection.

RESULTS

Zeb1 is highly expressed in the DN2 and DP stages of T cell development

To study the role of Zeb1 in T cell development, we sorted thymocyte subsets and measured Zeb1 transcript levels by semiquantitative (Q) RT-PCR (reverse transcription-polymerase chain reaction). As shown in Fig. 1a, Zeb1 transcript levels were low in DN1, began to increase in the DN2 stage and were maximal in DP thymocytes. Zeb1 expression then decreased as T cells underwent selection and matured into either conventional T cells or iNKT cells. Interestingly, the expression of Zeb2 was somewhat similar to that of Zeb1; high expression of Zeb2 was observed in early thymic progenitors (DN1–DN4), and the lowest expression of Zeb2 was observed in DP cells (Fig. 1b). This pattern of expression was corroborated by data from the ImmGen consortium²⁹ (Fig. S1A). Thus, as was observed in memory T cells,³⁰ Zeb1 and Zeb2 show similar patterns of expression in thymocytes. We used the ImmGen web browser to search for coregulated genes across different immune subsets. The E-protein Heb (encoded by *Tcf12*) was among the top 3 genes found to be coregulated with Zeb1 (Fig. S1B).²⁹ Heb is well known for its important roles throughout T cell development,³¹ especially in the DP stage,³² which further indicates that Zeb1 is a potential regulator of the DP developmental stage. We also analyzed the expression of Zeb1 protein in total thymocytes (80% of which consist of DPs). Zeb1 was strongly expressed in WT thymocytes but not in *Cellophane* thymocytes. Mutant mice expressed only reduced quantities of a truncated form of Zeb1 (Fig. 1c).

Impaired development of both conventional and unconventional T cells in *Cellophane* mice

To define the impact of the *Cellophane* mutation on T cell development, we analyzed the T cell composition in the thymus, spleen, and lymph nodes (LNs) of *Cellophane* mice. The T cell numbers in the spleen and liver were normal, while the number of lymphocytes was reduced in LNs (Fig. 2a). As shown by previous findings,²⁸ we also observed a strong decrease in the cell numbers in the thymus in *Zeb1*-mutated mice (Fig. 2a). This decrease in the cell number affected all subsets defined by CD4, CD8, CD44, and CD25 expression (Fig. 2b, c). The CD4⁺CD8⁺ DP thymocytes and DN2 populations also decreased in number within *Cellophane* thymocytes (Fig. 2b, c). In the LN and spleen, the percentages of

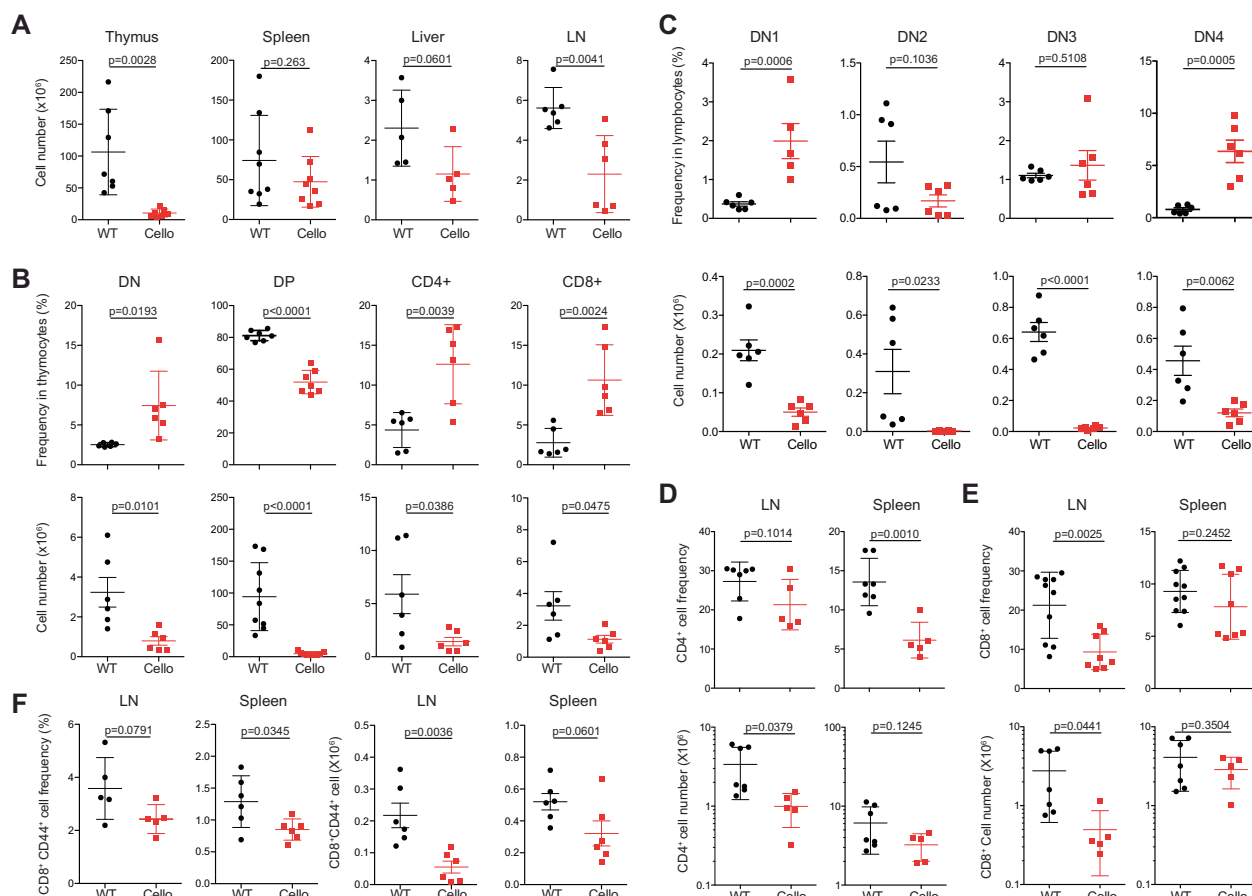


Fig. 2 Cellularity and proportions of T cell subsets in *Cellophane* mice. **a** Total number of cells from the thymus, spleen, liver, and LN. **b** Percentages and absolute numbers of the indicated T cell subsets (DN, DP, CD4 SP, and CD8 SP) in the thymus. **c** Percentages and absolute numbers of the indicated DN subsets defined by CD44 and CD25 expression in the thymus. Percentages and absolute numbers of **(d)** CD4 T cells and **(e)** CD8 T cells. **(f)** Percentages and absolute numbers of CD8⁺ CD44⁺ memory T cells in LN and spleen. Each dot represents an individual mouse. Data were pooled from 7 to 8 mice in four experiments (a–e) or three to six mice in two experiments (f). The statistical analysis was performed using an unpaired Student's *t* test

CD4 T cells and CD8 T cells were reduced (Fig. 2d, e), and the proportion of memory-phenotype CD44⁺ T cells among total CD8 T cells was decreased by nearly 30% (Fig. 2f).

We then investigated the development of unconventional T cell subsets. We observed a drastic decrease in the proportion and in the number of iNKT cells as well as NK1.1⁺ $\gamma\delta$ T cells in *Cellophane* mice compared with those in littermate controls (Fig. 3a–d). This decrease affected all organs in *Cellophane* mice (Fig. 3a–d). iNKT cells were mainly affected at stage 3 (Fig. 3e). To complete our analysis, we also studied memory-phenotype Ly49⁺ CD8 T cells, which are thought to arise “naturally” in the thymus without antigenic exposure.³³ All Ly49⁺ CD8 T cell populations were dramatically reduced in LN and spleen from *Cellophane* mice in terms of both proportions and numbers, irrespective of the inhibitory Ly49 receptor type that was analyzed (Ly49A, Ly49F, or Ly49G2) (Fig. 3f, g).

Altogether, these data confirm the important role of Zeb1 in early T cell development. We also demonstrated the essential and specific role of Zeb1 in the development of peripheral T cell subsets expressing NK cell markers, such as iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ CD8 T cells.

Intrinsic role of Zeb1 in thymic progenitors and T cell development
Zeb1 is also required for the development and expression of nonhematopoietic tissues and cell types.²⁰ To test whether Zeb1 played an intrinsic role in T cell development, we generated

chimeric mice by transplanting sublethally irradiated Ly5a (CD45.1) mice with BM from *Cellophane* (CD45.2) or “WT” Ly5a x C57BL/6 (CD45.1/2) mice. In the thymus of chimeric mice, the proportions of DN2 and DP cells were dramatically decreased in *Cellophane* BM-transplanted mice compared with those in WT BM-transplanted mice, while the proportions of other cell populations defined by CD4 and CD8 were increased (Fig. 4a). The iNKT and Ly49⁺ T cell subsets were also dramatically decreased in the peripheral organs of *Cellophane*→Ly5a BM chimeric mice compared with those of WT→Ly5a chimeric mice, indicating that Zeb1 intrinsically regulated T cell development (Fig. 4b, c). The number of cells in all thymic T cell subsets and the numbers of peripheral iNKT cells and Ly49⁺ T cells were decreased in *Cellophane*→Ly5a BM chimeric mice compared with those in WT→Ly5a chimera mice (Fig. S2). NK1.1⁺ $\gamma\delta$ T cells were not analyzed because many of them are derived from fetal precursors³⁴ and were not reconstituted in BM chimera mice. To further test the role of Zeb1 in the environment of developing T cells, we also generated different BM chimeric mice using WT and *Cellophane* mice as both BM recipients and donors (WT→WT, WT→*Cellophane*, *Cellophane*→WT, and *Cellophane*→*Cellophane*). As shown in Fig. S3A, B, the proportions and numbers of the DN and DP cells subsets were determined by the genotype of the BM donor rather than that of the recipient. Similar conclusions could be reached upon examination of the proportion and number of iNKT cells in the thymus and the liver (Fig. S3C).

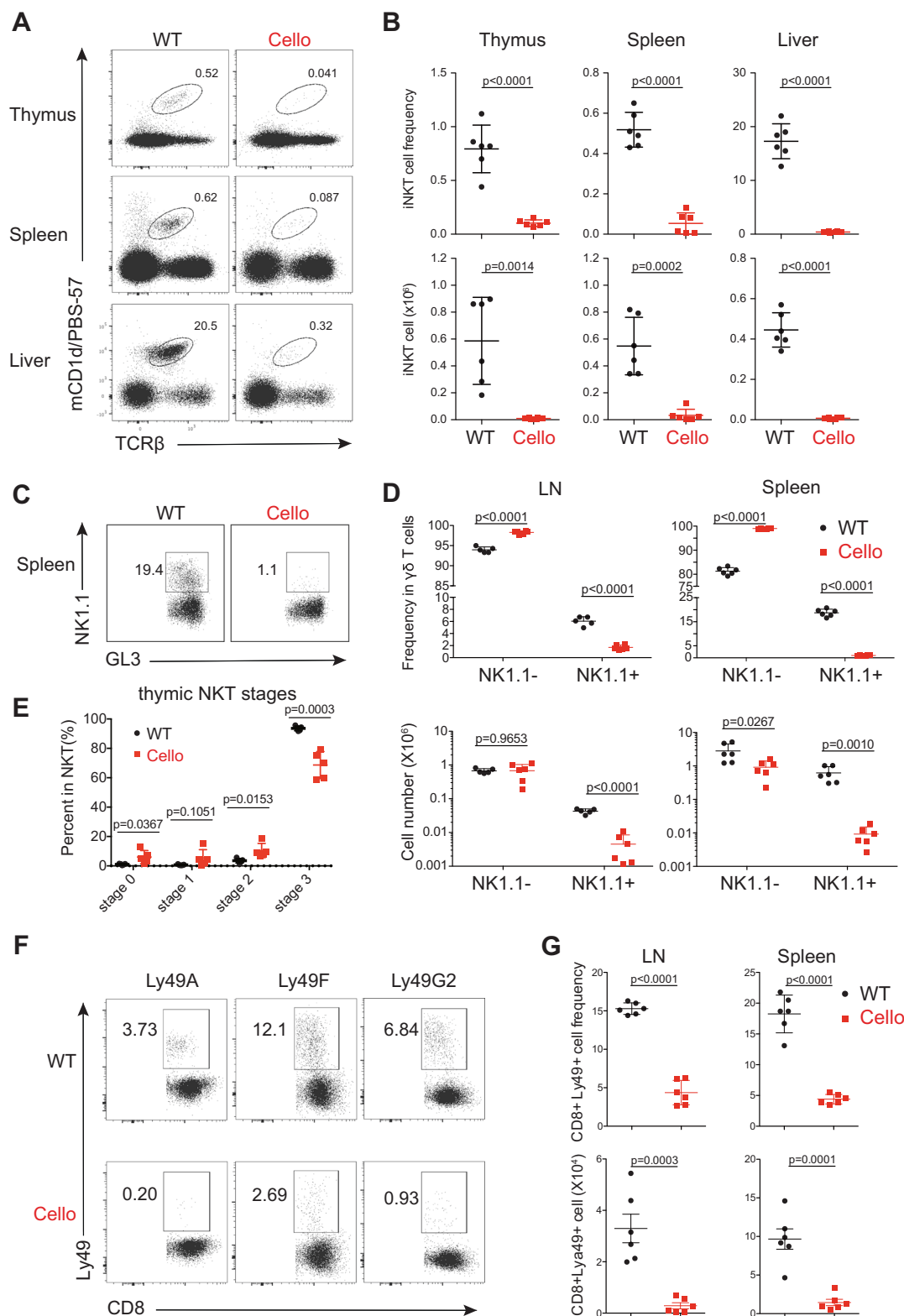


Fig. 3 *Cellophane* mice have decreased numbers of thymic DP cells and lack iNKT cells, NK1.1⁺ γδ T cells and Ly49⁺ CD8 cells. **a** Flow cytometry analysis of CD1d-tet⁺ iNKT cells (black gate) from thymus, spleen, and liver in wild-type (WT) and *Cellophane* (Cello) homozygous mice as indicated. **b** Percentages and absolute numbers of iNKT cells (mCD1d/PBS-57⁺ TCRβ⁺) in the thymus, spleen, and liver. **c** Flow cytometry analysis of NK1.1⁻ and NK1.1⁺ γδ T cells (black square gates) from the spleen of WT and *Cellophane* mice. **d** Percentages within total γδ T cells and absolute numbers of NK1.1⁻ and NK1.1⁺ γδ T cells in spleen and LN. **e** Percentage within total thymic iNKT cells of cells from stages 0–3, as defined by CD24, CD44, and NK1.1 expression. **f** Flow cytometry analysis of CD8⁺ CD44⁺ Ly49A⁺/Ly49F⁺/Ly49G2⁺ T cells (black square gates) from LN and spleen from WT and *Cellophane* mice. **g** Percentage within CD8⁺ CD44⁺ T cells and absolute number of CD8⁺ CD44⁺ Ly49⁺ T cells in spleen and LN. Each dot represents an individual mouse. Data are representative of two to four independent experiments with six mice in total in each panel. The statistical analysis was performed using an unpaired Student's *t* test

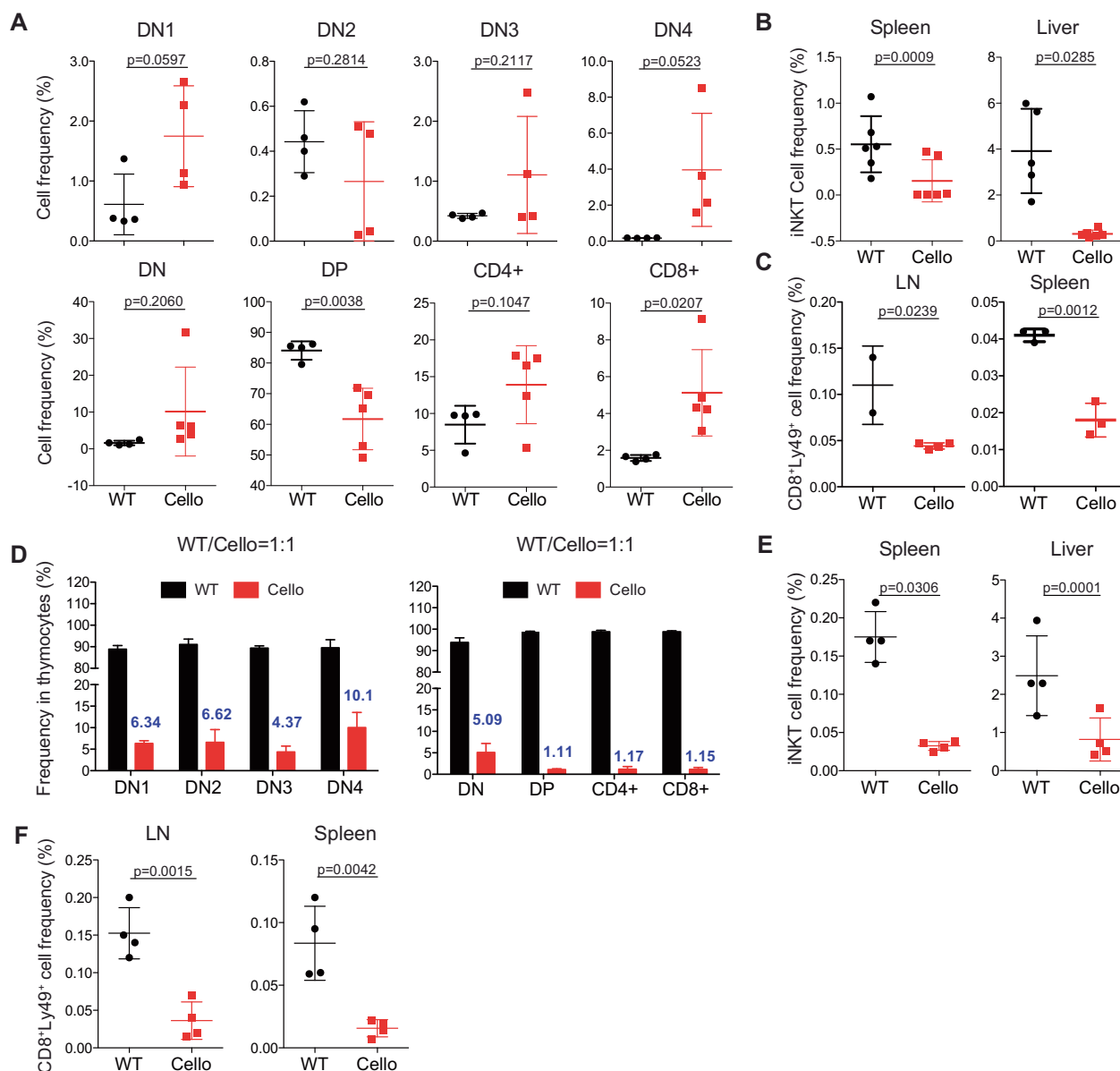


Fig. 4 The role of Zeb1 in T cell development is cell-intrinsic. **a** Flow cytometry analysis of the indicated thymocyte subsets from BM chimeras. Frequencies are shown for each subset. Recipients were Ly5a mice (CD45.1), and the donor BM was obtained from either Ly5a x C57BL/6 (CD45.1/2) or *Cellophane* (CD45.2) mice. **b** iNKT cell and **c** CD8⁺ Ly49⁺ T cell frequencies in the spleen, liver, or LN from the same BM chimera described in **a**. **d** DN1-4, DP, CD4⁺, and CD8⁺ cell reconstitution in the thymus of competitive BM chimeras. Recipients were Ly5a mice (CD45.1), and the donor BM was a 1:1 mix of cells from Ly5a x C57BL/6 (CD45.1/2) and *Cellophane* (CD45.2) mice. Eight weeks after BM transplantation, the cells were analyzed by flow cytometry. The number above the red bar shows the percentage of the indicated cells originating from *Cellophane* BM. **e, f** Frequencies of iNKT cells (**e**) and Ly49⁺ cells within CD8⁺ CD44⁺ T cells in spleen, liver or LN from chimera BM described in **d**. Each dot represents an individual mouse. The results show the pooled data from two to three independent experiments for a total of two to six mice per group in each panel. Statistical analyses were performed using an unpaired Student's *t* test

We then generated mixed BM chimeras by transplanting lethally irradiated Ly5a mice with a 1:1 mixture of BM from *Cellophane* and Ly5a x C57BL/6 (WT) mice.

Cellophane T cell progenitors showed poor competitive fitness in BM chimeric mice (Fig. 4d). Indeed, the percentage of cells originating from the *Cellophane* BM progenitors was already low in the DN stage and further decreased during the transition between the DN and DP stages (Fig. 4d). In the periphery of mixed BM chimeric mice, we found that the proportions of iNKT and Ly49⁺ CD8 T cells were greatly reduced among *Cellophane* T cells compared with those among WT lymphocytes (Fig. 4e, f), thus revealing that the role of Zeb1 in T cell development is cell-intrinsic and is not due to a defective stromal environment. Of

note, we also analyzed the reconstitution of myeloid cells as a control. In the spleen, on average, 20% of macrophages, 25% of dendritic cells, and 28% of neutrophils originated from *Cellophane* mice (Fig. S3D), suggesting that Zeb1 regulated the development of all hematopoietic subsets, perhaps by regulating multipotent progenitors. However, the most important effects were observed for thymocytes and peripheral T cell subsets expressing NK cell markers.

Reduced survival and proliferation of *Cellophane* DN2 and DP cells
The decreased cellularity of the *Cellophane* thymi could be due to the reduced proliferation or increased apoptosis of thymocytes. To address this point, we first compared the survival of WT and

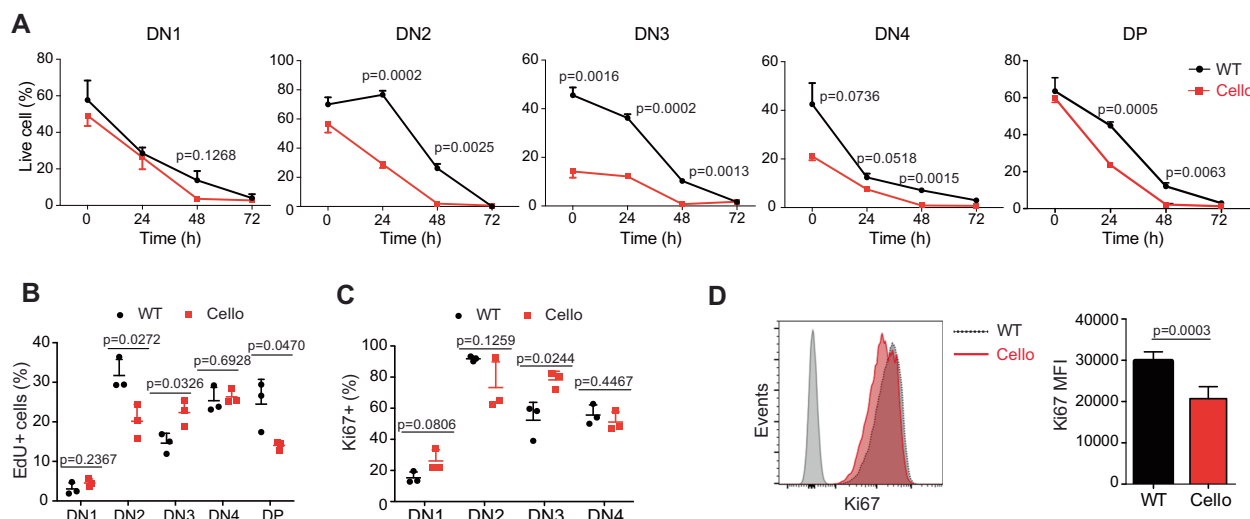


Fig. 5 *Cellophane* DP cells show reduced survival and proliferation. **a** Percentages of annexin-V-negative cells (live cells) among wild-type and *Cellophane* thymocytes cultured for 0–72 h. Each graph shows a different subset, as indicated. **b** EdU incorporation in wild-type and *Cellophane* thymocytes after a 12-h in vivo pulse of 0.2 mg EdU. **c** Frequencies of Ki67⁺ cells in DN1–4 cell subsets from WT and *Cellophane* mice. **d** Ki67 nuclear staining in DP thymocytes from WT and *Cellophane* mice. Bar graphs (right panel) show the mean \pm SD fluorescence intensity (MFI). Each dot represents an individual mouse. Data are pooled from three independent experiments with three mice in each group (**a–d**). Statistical analysis was performed using an unpaired Student's *t* test

Cellophane thymocytes during ex vivo culture. We found that *Cellophane* thymocytes in DN2, DN3, and DN4 showed reduced ex vivo viability compared with their WT counterparts (Fig. 5a). Moreover, after 24 or 48 h in culture, *Cellophane* DN2, DN3, DN4, and DP cells also showed reduced viability compared with control cells (Fig. 5a).

Next, we compared the in vivo proliferation of WT and *Cellophane* thymocytes, as measured by EdU incorporation. *Cellophane* DN2 and DP cells showed less proliferation than their WT counterparts, while *Cellophane* cells proliferated more than WT DN3 cells (Fig. 5b). Ki67 staining corroborated the data we obtained by using EdU incorporation (Fig. 5c, d). As all DP cells were Ki67 positive, we only reported the changes in the mean fluorescence intensity (MFI) (Fig. 5d).

Thus, the *Cellophane* mutation affects both the survival and proliferation of developing DN2 and DP thymocytes, which could account for the decreased number of such cells in the *Cellophane* thymi.

Zeb1 modulates TCR signaling strength

To gain insight into the mechanism of Zeb1 function, we focused on DP cells, as they expressed the highest level of Zeb1 among thymocytes (Fig. 1a). We first compared the expression of membrane proteins involved in thymocyte selection in *Cellophane* versus WT DP cells by flow cytometry. *Cellophane* DP cells expressed higher levels of Cd69, Cd25, and Cd5 than WT thymocytes (Fig. 6a). Nur77 is an early response gene expressed in T cells within hours after TCR stimulation. We observed an increase in the intracellular expression of Nur77, which was more highly correlated with Tcr β levels in *Cellophane* cells than in WT DP thymocytes (Fig. 6a). Notably, similar levels of Cd4 and Cd8 were detected (Fig. 6a).

Next, we focused our attention on thymic iNKT cell subsets. The mouse thymi is known to contain at least three iNKT subsets, iNKT1, iNKT2, and iNKT17, which are thought to play distinct roles in the immune response.³⁵ iNKT1 cells comprise mainly stage 3 iNKT cells. TCR signaling strength governs the development of iNKT cell subsets in the thymus, in which high signaling strength is necessary for iNKT2 and iNKT17 development.^{9,10} We examined iNKT cell subsets by staining for Plzf and Ror γ ³⁶ in *Cellophane* mice and WT mice. The results in Fig. 6b show a significant increase in

the proportions of iNKT2 and iNKT17 cells and a decrease in the proportion of iNKT1 cells in *Cellophane* mice compared with those in control mice. These data indicated an increase in the TCR signaling from DP progenitors of iNKT cells in *Cellophane* mice. The change in iNKT subsets was associated with subtle changes in the TCR repertoire, as assessed by measuring the frequencies of V β 8-, V β 7-, and V β 2-positive cells among iNKT cells of each genotype. We observed a twofold increase in V β 7 in *Cellophane* mice (Fig. 6c). This could reflect the increase in iNKT2 cells, as a previous study showed that V β 7 was more often associated with iNKT2 cells.⁹ Thymic *Cellophane* iNKT cells expressed normal levels of the TFs T-bet and Egr2 but strongly reduced levels of Cd4 (Fig. 6d). Since CD4 is known to sustain TCR signaling strength,⁵ the selection of CD4^{low} iNKT cells in *Cellophane* mice could reflect the adaptation to overt TCR signaling in *Cellophane* DP cells.

We then specifically analyzed TCR signaling in developing thymocytes. We started by measuring the phosphorylation (p) levels of a series of signaling proteins involved in TCR-mediated activation, either at the steady-state in freshly isolated thymocytes or following TCR engagement by cross-linking with anti-CD3 antibodies. To minimize the experimental variation, we used a barcoding strategy that allowed stimulation and then staining of WT and *Cellophane* thymocytes simultaneously (see “Materials and methods”). The results in Fig. 6e, f show increases in pAkt (Ser473), ribosomal protein pS6, and, to a lesser extent, pErk either at the steady-state or following TCR engagement in *Cellophane* DP cells compared with WT DP cells. Thus, the MAPK and PI3K/Akt pathways are more active in thymocytes undergoing selection in *Cellophane* mice compared with those in control mice. To complement this analysis, we also assessed the calcium response of DP cells of both genotypes in response to TCR engagement. As shown in Fig. 6g, h, this response was stronger for *Cellophane* DP cells than control DP cells in terms of both intensity (peak) and duration (area under the curve, AUC). Thus, Zeb1 modulates the signaling strength downstream of the engaged TCR at the DP stage, and *Cellophane* DP cells show increased TCR signaling, which may increase negative selection and therefore account for defective T cell development in *Cellophane* mice. We also measured pre-TCR signaling in DN cells following stimulation with anti-CD3 antibodies. This analysis did not reveal any difference between WT and *Cellophane* DN cells (data not shown).

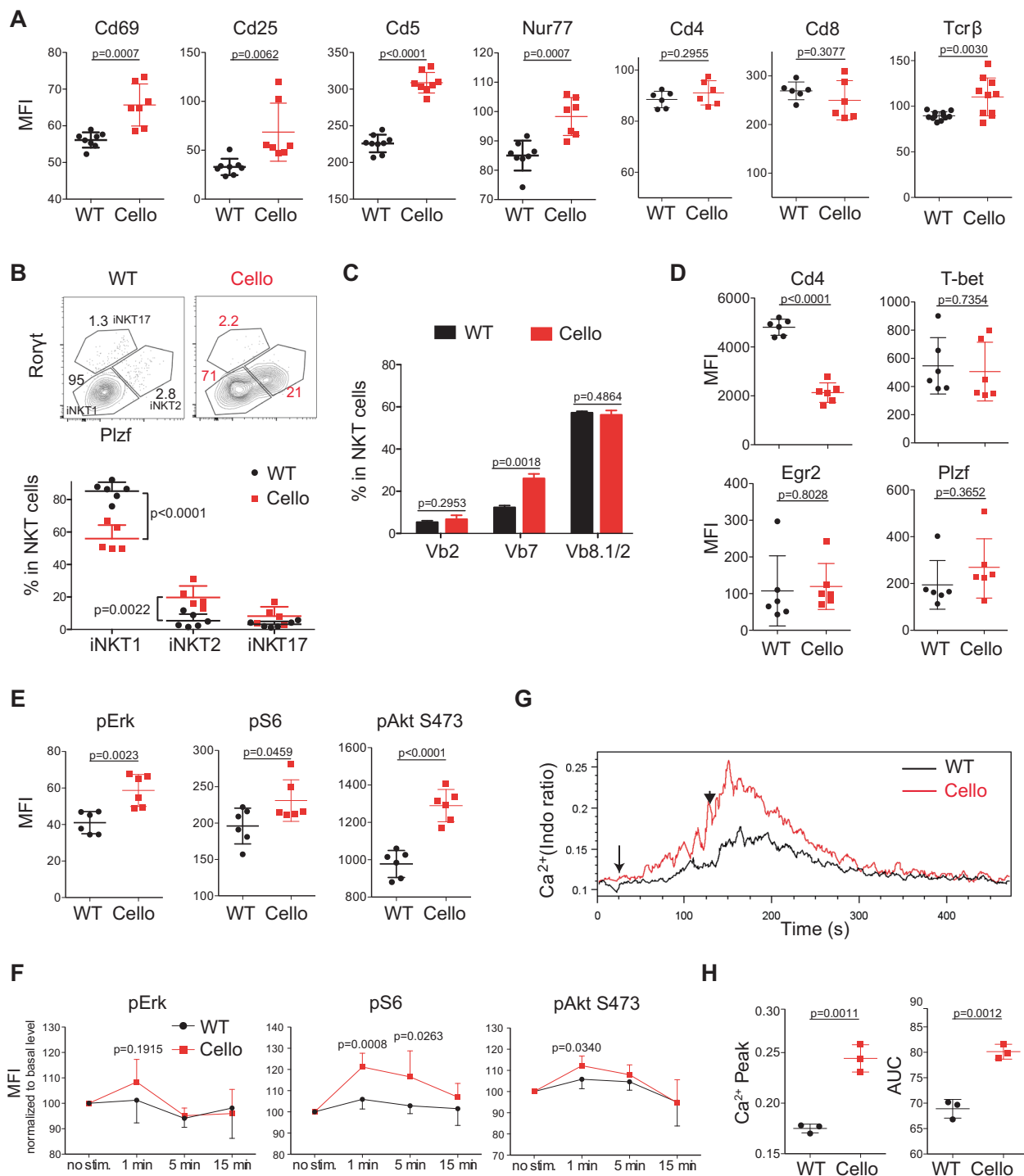


Fig. 6 *Cellophane* DP thymocytes display increased TCR signaling. **a** Flow cytometry analysis of Cd69, Cd25, Cd5, Nur77, Cd4, Cd8, and Tcrβ expression (mean fluorescence intensity) in DP thymocytes of wild-type and *Cellophane* mice. **b** Left panels: Representative flow cytometry analysis of iNKT1 (Plzf lo, Roryt-, T-bet+), iNKT2 (Plzf hi, Roryt-, T-bet-), and iNKT17 (Plzf int, Roryt+, T-bet-) cell subsets in wild-type and *Cellophane* mice (as defined by the gating strategy shown in the upper panel). Right panel: Graphs of the percentages of iNKT cell subsets within total iNKT cells. **c** The TCRVβ repertoire of thymic iNKT cells was analyzed by flow cytometry. Bar graphs show the mean ± SD frequencies of individual Vβ chains within iNKT cells. **d** Expression of Cd4, T-bet, Egr2, and Plzf in iNKT cells as measured by flow cytometry (mean fluorescence intensity). **e** Phosphorylation levels of Erk, S6, and Akt (S473) in resting DP thymocytes from WT and *Cellophane* mice. **f** Phosphorylation levels of Erk, S6, and Akt (Ser473) in DP cells stimulated with anti-CD3 antibody for the indicated times. Results are normalized to the nonstimulated condition (i.e., basal level is 100%) for each group. **g** Representative histogram overlay showing the Ca²⁺ flux in thymic DP cells from wild-type or *Cellophane* mice stimulated with anti-CD3 antibodies, as measured by flow cytometry. Thymocytes were activated following incubation with biotinylated anti-CD3 (arrow) followed by cross-linking with streptavidin (arrowhead). **h** Dot plots showing the AUC (area under the curve) and Ca²⁺ peak. Statistical analysis was performed using an unpaired Student's *t* test. Each dot represents an individual mouse. Data are pooled from three experiments with a total of eight mice in each group (**a**), three experiments with six mice (**b**), three mice (**c**), two experiments with six mice (**d–f**) or three experiments with three mice (**g, h**)

Zeb1 broadly shapes transcription during the DP → SP transition to promote proliferation and repress TCR signaling

To further uncover the mechanisms of Zeb1 function during T cell development, we performed RNA-seq to compare the WT and *Cellophane* DP transcriptomes. We found 538 differentially expressed genes (DEGs, p value < 0.05, log₂-fold change > 1). A total of 204 genes were increased, and 334 were downregulated in *Cellophane* DP cells compared with WT DP cells. These data reveal that Zeb1 broadly shapes the genetic program of developing thymocytes (Fig. 7a and Table S1). For some of the DEGs identified, antibodies were available, and we were thus able to confirm the higher expression of Foxo1, Ms4a4b, Itgb7, Ccr7, and Ccr4 in *Cellophane* cells compared with that in control DP cells; in contrast, we observed lower Cd81 expression in *Cellophane* DP cells than in WT cells (Fig. 7b).

Next, we queried the ImmGen database to retrieve the expression profile of the Zeb1-regulated gene set (induced or repressed) across all thymocyte subsets. Interestingly, genes downregulated in *Cellophane* DP cells (i.e., normally induced by Zeb1) correspond to genes that are normally expressed at high levels in early T cell progenitors and at low levels in mature T cells (Fig. 7c). Their expression level normally drops during the DP to SP transition, which is when Zeb1 is highly expressed. Genes upregulated in *Cellophane* DP cells correspond to genes that show a reciprocal pattern of expression (Fig. 7c). This pattern of expression also correlates with cell proliferation and TCR responsiveness in thymocytes. Indeed, irrespective of the mouse genotype, SP T cells are much more responsive to TCR signaling-mediated calcium responses than DP cells but also do not cycle as much as DP cells (data not shown). This suggests that Zeb1 promotes cell proliferation and represses TCR signaling specifically at the DP stage, presumably to ensure proper selection.

A functional annotation analysis of DEGs identified by our RNA-seq analysis using “Metascape”³⁷ highlighted the cell cycle as the most downregulated biological process in *Cellophane* DP cells compared with control DP cells (Fig. 7d and Table S2), confirming the findings in Fig. 3. Pathways linked to Ifn γ (and also type I-Ifn; Table S2), antigen presentation, leukocyte differentiation, and apoptosis were significantly associated with genes upregulated in *Cellophane* DP cells compared with those in WT cells (Fig. 7d). Of note, a modest but significant enrichment of genes involved in the calcium response was also associated with these upregulated genes, corroborating the data shown in Fig. 6. To further annotate this dataset, we performed individual PubMed searches to look for connections between genes upregulated in *Cellophane* DP cells compared with control DP cells and “T cell activation”, “TCR signaling”, or “T cell development”. Interestingly, this analysis showed that more than 25% of the genes in the list played a known role in T cell activation or TCR signaling, and 10% played a role in T cell development, as defined using loss-of-function mouse strains (Table S3). Moreover, we used the STRING database of physical and functional protein interactions³⁸ to further annotate genes that were up- or downregulated in *Cellophane* DP cells compared with control DP cells. In particular, we used the PubMed module that searches for the enrichment of gene lists in articles in PubMed. This unbiased analysis showed that genes upregulated in *Cellophane* DP cells were significantly enriched for genes involved in negative selection³⁹ or T cell maturation regulated by Bcl11b⁴⁰ (Table S4), which corroborated our manual PubMed searches.

Chromatin regions remodeled at the DP stage contain Zeb1 binding motifs

Next, we wanted to determine whether Zeb1 could regulate chromatin remodeling at the DP stage of T cell development. For this, we took advantage of a recently published large-scale analysis of chromatin accessibility and gene expression across 86 immune cell subsets, including subsets representing T cell

developmental stages in the thymus.⁴¹ In this study, in silico predictions identified Zeb1 as one of the few TFs whose expression was correlated with modifications of chromatin accessibility during thymic T cell development and for which the corresponding chromatin regions contained sites predicted to be bound by Zeb1. Other TFs in this category included Gata3, Tcf7, Lef1, Tcf12, and Zfp740 (Fig. S4A), especially Tcf7 and Tcf12, whose roles in T cell development have been well established.³² We retrieved the open chromatin regions (OCRs) for which Zeb1 motifs were discovered in this study and whose accessibility changed during T cell development (see the corresponding clusters in Fig. S4B) and compared the list of corresponding genes with the DEGs between *Cellophane* and WT DP cells identified in our own study. We found an important overlap between both lists that included many of the genes previously highlighted in our analysis (Fig. 7e, p value = 1.839413e–46). Altogether, these data suggest that Zeb1 is a direct transcriptional regulator of T cell development that is especially involved in the DP to SP transition that promotes cell proliferation and ensures proper selection.

DISCUSSION

Here, we demonstrated that Zeb1 is essential for the transition through the DN2 and DP stages of T cell development as well as for the differentiation of iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ CD8 T cells. Mechanistically, Zeb1 regulates the expression of a number of genes that are notably involved in cell proliferation or in TCR signaling at the DP stage. In *Cellophane* mice, these events may perturb thymic development and selection in a way that does not allow the production of the NK1.1⁺ and Ly49⁺ T cell subsets.

Zeb1 expression was found to increase at the DN2 stage and to be maximal at the DP stage of T cell development. Accordingly, we found decreases in the frequencies of DN2 and DP thymocytes in *Cellophane* mice. This could be due to the cell-intrinsic role of Zeb1 in DN2 and DP proliferation. A number of genes involved in the cell cycle were differentially expressed between WT and *Cellophane* DP cells, as revealed by our RNA-seq analysis. There is also a strong link in the literature between Zeb1 and cell proliferation in cancer. In particular, Zeb1 interacts with many TFs involved in the regulation of cell growth, such as Smad TFs, which are downstream of several growth factor pathways.²² Moreover, Zeb1 is known to repress cyclin-dependent kinases during EMT.²¹ However, the levels of CDkn2c and CDkn3 were decreased by Zeb1 in DP thymocytes, suggesting the different roles of Zeb1 in epithelial versus lymphoid cells. The decreased proliferation of DN2 and DP cells is expected to have important consequences for overall thymic output. Indeed, we found decreased numbers of peripheral T cells in *Cellophane* mice. However, this defect was much more pronounced for iNKT cells, NK1.1⁺ $\gamma\delta$ T cells, and Ly49⁺ CD8 T cells. In particular, iNKT cells were virtually absent from the periphery. The altered development of T cells was associated with increased TCR signaling at the DP stage, which was verified by increased basal levels of Cd5 and Nur77 and increased mTOR activity and calcium flux upon CD3 engagement. iNKT cells are known to receive stronger TCR signals than conventional T cells during their development.⁶ Thus, increased TCR signaling in *Cellophane* DP cells could trigger cell death via negative selection of iNKT precursors. The increased negative selection of iNKT cells has already been shown to occur in mice in which transgenic TCR- β chains confer high affinity for self-lipid/CD1d complexes when they are randomly paired with Va14-Ja18 rearrangements.⁴² Thus, increased negative selection could impair the development of iNKT cells in *Cellophane* mice and perhaps that of other T cell subsets expressing NK cell markers. Indeed, strong TCR-mediated signals are also important for $\gamma\delta$ T cell development.^{43,44} In particular, NK1.1⁺ $\gamma\delta$ T cells have an oligoclonal TCR repertoire and accumulate in mouse models of decreased TCR signaling,⁴⁵ suggesting that this subset of $\gamma\delta$ cells

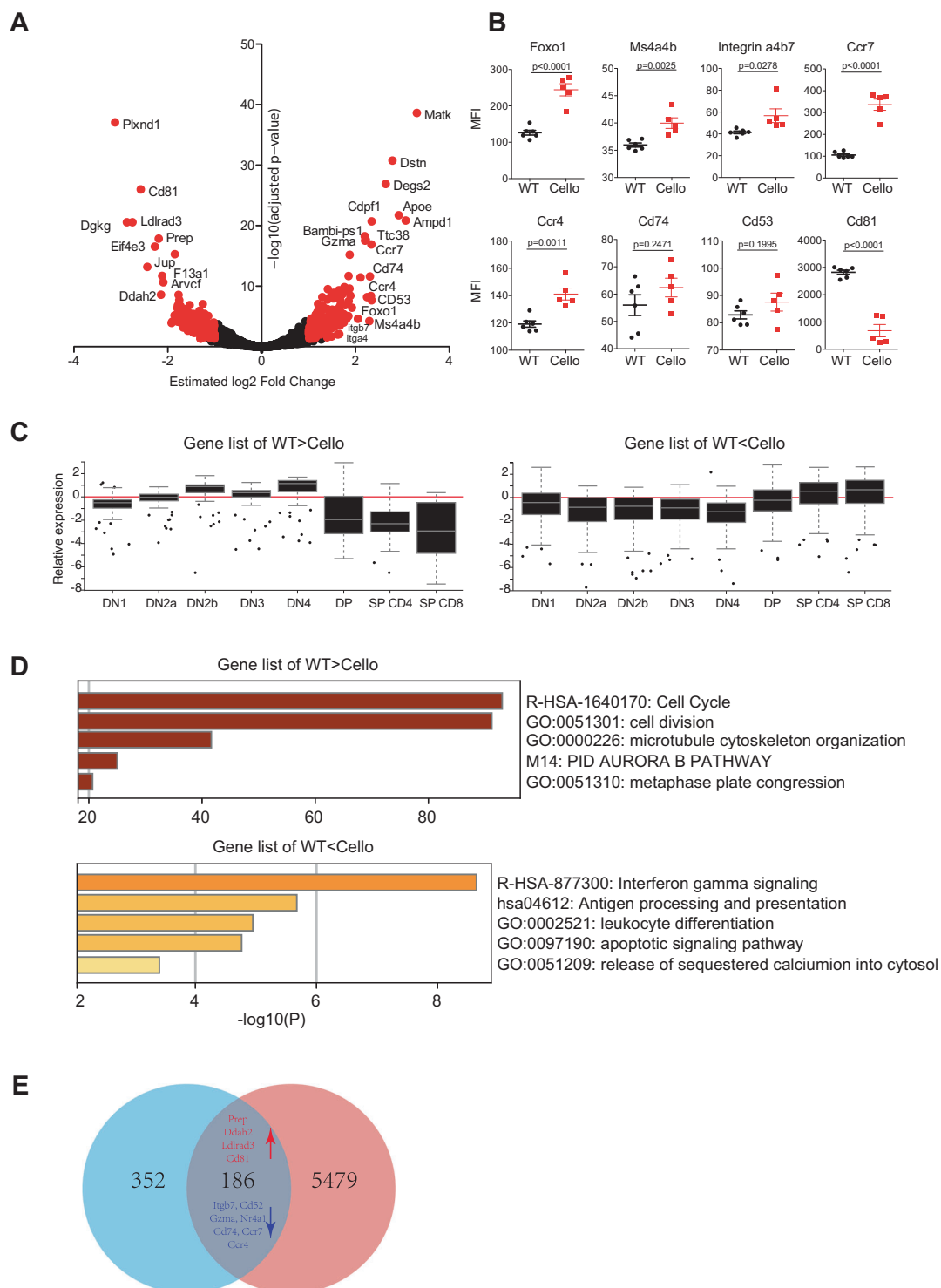


Fig. 7 RNA-seq analysis of *Zeb1*-regulated genes. **a** Volcano plot representing the $-\log_{10}$ adjusted p value as a function of the estimated \log_2 -fold-change. Significant genes were selected using the following thresholds: an adjusted p value lower than 0.05 and an absolute value of the \log_2 -fold-change greater than 1. For significant genes (plotted in red), a selection of the gene names is displayed. The results are representative of the biological replicates in each group. **b** Flow cytometry analysis of several genes identified by RNA-seq as either upregulated (Foxo1, Ms4a4b, integrin $\alpha 4:\beta 7$, Ccr7, Ccr4, Cd74, and Cd53) or downregulated (Cd81) in *Cellophane* mice. Data are pooled from two independent experiments with a total of six mice. **c** Expression level of genes in two selected lists (200 genes in each list) that were either upregulated (WT < Cello) or downregulated (WT > Cello) in different $\alpha\beta$ T cell subsets in *Cellophane* mice with a fold change > 2 and a p value < 0.05, as indicated. Graphs are adapted from the ImmGen website. **d** Pathway analysis of DEGs in **a** using Metascape. The selected terms are shown among the most significant terms. **e** Venn diagram showing the overlap between DEGs in **a** and genes located within 100 kb of OCRs containing *Zeb1* motifs for which chromatin accessibility changed during T cell development (the corresponding clusters are visualized in Fig. S4B). The genes upregulated in WT mice are shown in red, while the genes that were downregulated are shown in blue

can also be negatively selected. The ontogeny of Ly49⁺ CD8 T cells is not very well known, but our data suggest that their development and selection could share common mechanisms with those of NK1.1⁺ T cells. How does Zeb1 regulate TCR signaling strength? *Cellophane* DP thymocytes expressed higher levels of Tcr β than control DP thymocytes, and this could certainly lead to increased TCR signaling. Moreover, the RNA-seq analysis we performed suggested that there were multiple connections between Zeb1 and signaling transduction through the TCR. For example, multiple members of the GTPases of the IMMunity-Associated Proteins (GIMAP) family (GIMAP5 and GIMAP8) were upregulated in *Cellophane* DP cells. Interestingly, Gimap5 enhances calcium influx following TCR stimulation.⁴⁶ Several members of the Ms4a family of receptors, which have four transmembrane domains, are also upregulated in *Cellophane* DP cells and could reinforce TCR signaling. Indeed, the transduction of signaling by Ms4a4b in naive T cells can heighten their sensitivity to antigens through a process that could involve association with costimulatory molecules.⁴⁷ Several phosphatases and kinases are also deregulated in *Cellophane* DP cells and could perhaps alter TCR signaling. In particular, the expression of Pyk2 (encoded by *Ptk2b*), Rasgrp4, Rasl1, or Rasa3 could all contribute to increased TCR signaling via calcium flux or the MAPK pathway.

A series of TFs were also deregulated in *Cellophane* DP cells, which showed notable upregulation of JunB, Jun, Atf6, Foxo1, Stat4, or Irf7/9. JunB and Jun are essential components of AP-1 TFs and are typically activated downstream of TCR stimulation. Similar to Nur77, they could represent surrogate markers of increased TCR signaling in *Cellophane* DP. The derepression of Foxo1 could in part account for altered T cell development, as it regulates Ccr7, CD62L, and S1pr1 via Klf2.⁴⁸ The lack of control by Klf2 could perturb and perhaps even accelerate the normal migration of developing thymocytes in the medullary region where negative selection occurs. Foxo1 deletion in thymocytes was reported to decrease the number of DP thymocytes, and Foxo1-deficient peripheral T cells seem to be refractory to TCR stimulation through unknown mechanisms.⁴⁹ Moreover, upregulation of Foxo1 in *Cellophane* DP cells could in part explain the inverse changes in cell proliferation observed in these cells compared with those in control DP cells, since Foxo TFs are known to promote stem cell quiescence⁵⁰ and clearly contribute to the regulation of cell division, survival, and metabolism in T cells.⁵¹ A recent study showed that the transcriptional repressor Gfi1 is important in maintaining Foxo1 expression at low levels in DP thymocytes.⁵² In the absence of Gfi1, premature expression of genes normally expressed in mature T cells and accelerated maturation of DP cells into SP thymocytes occurred, which was largely attributable to Foxo1 derepression. Zeb1 and Gfi1 could therefore cooperate to repress Foxo1.

There are many similarities in the phenotypes of *Tcf12*-deficient⁵³ and *Cellophane* mutant mice, particularly in terms of the susceptibility of DP cells to cell death and the impaired development of iNKT cells. Moreover, microarray data from the ImmGen consortium suggest that *Tcf12* and Zeb1 are strongly coregulated, and ATAC-seq data predict that they control chromatin accessibility during thymic T cell development together with Gata3, Tcf1, Lef1, and Zfp740 (Fig. S4 and ref. 41). Altogether, these data suggest a strong functional link between Zeb1, Heb (encoded by *Tcf12*), and perhaps Tcf1, which acts in coordination with Heb.³² The fact that Zeb1 is known to bind tandem E-box motifs suggests that there is possibly competition between Zeb1 members and E proteins for binding of those genes regulated by tandem E-boxes. Such competition has been previously established in the context of the CD4 enhancer, which is repressed by Zeb1, through competition with Heb for E-box binding.⁵⁴ Moreover, Zfh-1 and Daughterless, the *Drosophila* homologs of Zeb1 and *Tcf12*, are also known to compete for the same genomic sites.⁵⁵ The deletion of *Tcf12* and *Tcf1* in thymocytes results in the opposite phenotype as the *Cellophane* Zeb1 mutation in terms of

DP proliferation.³² This suggests that Heb and Zeb1 could have partially antagonistic activities in the regulation of genes bearing tandem E-box elements. Competition between Zeb1 and E proteins has already been suggested to play a role in the control of GATA3 expression in human CD4 T cells.⁵⁶ *Cellophane* mice express a truncated form of Zeb1 that is expressed at lower levels than WT Zeb1. As the phenotype of these mice is milder than that of *Zeb1*^{-/-} mice, we assumed that the *Cellophane* mutation was hypomorphic. However, we cannot rule out that *Cellophane* Zeb1 may retain some DNA-binding capability and therefore act as a dominant negative molecule by preventing the binding of E-box proteins. Further work will be needed to precisely map the interactions between Zeb TFs and E proteins. The regulatory network may also include inhibitors of the differentiation genes *Id2* and *Id3*, which are TFs that bind and inactivate E proteins, thereby regulating their function. Moreover, a deficiency in *Id3* has the same impact on NK1.1⁺ $\gamma\delta$ T cells as a deficiency in TCR signaling,⁴⁵ which indicates a link between both factors.

The *Zeb1* genomic region is frequently deleted in cutaneous T cell lymphomas (CTCLs).⁵⁷ Such deletions are often associated with genetic mutations in components of the TCR signaling machinery (recurrent alterations in *Card11*, *Plcg1*, *Lat*, *Rac2*, *Prkcd*, *CD28*, and genes that encode calcium channel subunits). This observation, together with our own data showing the role of Zeb1 in repressing TCR signaling, suggests that Zeb1 deletion could promote lymphomagenesis by releasing the normal constraints on TCR signaling. Of note, a previous study proposed the essential role of IL-15 in CTCL development and showed that IL-15 expression was suppressed in patients with CTCL due to promoter hypermethylation and the failure of Zeb1 to gain access to and repress the IL-15 regulatory region.⁵⁸ However, IL-15 expression was not detected in developing thymocytes in our RNA-seq analysis, excluding the possibility that the IL-15 pathway could play a role in the mechanism of action of Zeb1 in T cell development. However, we found that the transcriptional responses to different cytokines, such as interferons or IL-6, were increased in *Cellophane* DP cells (Table S2). Zeb1 may therefore normally repress the responses to these cytokines, which presumably occurs to ensure proper selection. TGF- β is a known regulator of iNKT cell development⁵⁹ that promotes early differentiation and prevents the apoptosis of developing iNKT cells. A recent study showed that Zeb1 expression was induced by TGF- β in conventional CD8 T cells stimulated through the TCR and was essential for memory T cell survival and function.³⁰ Although we failed to detect any effect of recombinant TGF- β on Zeb1 expression in thymocytes (data not shown), it would be interesting to address this question in vivo using appropriate genetic models.

In summary, Zeb1 is an essential member of the TF network that regulates T cell development and selection in the DN2 and DP stages. Furthermore, we have also shown that Zeb1 facilitates the development of iNKT cells and other T cell subsets expressing NK cell markers by regulating the cell cycle and TCR signaling in developing thymocytes.

MATERIALS AND METHODS

Mice

Mice that were 8–24 weeks old were used. Wild-type C57BL/6 mice were purchased from Charles River Laboratories (L'Arbresle). *Cellophane* mice were previously described,²⁸ and their littermates were used as controls. This study was carried out in accordance with the French recommendations in the Guide for the Ethical Evaluation of Experiments Using Laboratory Animals and the European guidelines 86/609/CEE. All experimental studies were approved by the local bioethics committee CECCAPP. Mice were bred at the Plateau de Biologie Expérimentale de la Souris (ENS, Lyon).

Bone marrow chimeric mice

8- to 10-week-old Ly5a mice or Ly5a x C57BL/6 mice were anesthetized with ketamine/xylazine before irradiation at a dose of 9 Gray with an X-ray irradiator XRAD-320. After irradiation, they were intravenously injected with $2-5 \times 10^6$ cells collected from either wild-type or mutant murine bone marrow or a mix of both (as indicated in the figures). Immune cell reconstitution was analyzed 8 weeks post BM injection.

Flow cytometry

Single-cell suspensions of thymus, spleen, and liver were used for flow cytometry. Cell viability was measured using annexin-V (BD Biosciences)/live-dead fixable (eBiosciences) stain. Intracellular staining for TFs was performed using a Foxp3 kit (eBioscience). Lyse/Fix and PermIII buffers (BD Biosciences) were used for intracellular staining of phosphorylated proteins. Flow cytometry was carried out on a FACS Canto, a FACS LSRII, or a FACS Fortessa (Becton-Dickinson). Data were analyzed using FlowJo (Treestar). Antibodies were purchased from eBioscience, BD Biosciences, R&D Systems, Beckman Coulter, Miltenyi, or Biolegend. We used the following antibodies: anti-mouse CD3 (clone 145-2C11), anti-mouse CD4 (clone GK1.5), anti-mouse CD8 (clone 53-6.7), anti-mouse TCR β (clone H57-597), anti-mouse CD69 (clone H1.2F3), anti-mouse TCR $\gamma\delta$ (clone GL3), anti-mouse NK1.1 (clone PK136), anti-mouse CD24 (clone M1/69), anti-mouse CD44 (clone IM7), anti-mouse CD27 (clone LG.7F9), anti-mouse TCRV β 2 (clone B20.6), anti-mouse TCRV β 7 (clone TR310), anti-mouse TCRV β 8.1/8.2 (clone KJ16-133), anti-mouse Ly49A (clone A1), anti-mouse Ly49E/F (clone REA218), anti-mouse Ly49G2 (clone 4D11), anti-mouse CD45.1 (clone A20), anti-mouse CD45.2 (clone 104), anti-mouse Nur77 (clone 12.14), anti-mouse Ccr7 (clone 4B12), anti-mouse CD5 (clone 53-7.3), anti-mouse CD81 (clone Eat-2), anti-mouse CD53 (clone OX79), anti-mouse Lpam-1 (clone DATK-32), anti-mouse Foxo1 (clone C29H4), anti-mouse Ms4a4b (clone 444008), anti-mouse CD74 (clone In1/CD74), anti-mouse T-bet (clone 4B10), anti-mouse Egr2 (clone erongr2), anti-mouse Plzf (clone Mags.21F7), anti-mouse Ror γ t (clone AFKJS-9), anti-mouse pErk (clone 20A), anti-mouse pAkt (Ser473) (clone M89-61), and anti-mouse pS6 (clone D57.2.2E). For the staining of iNKT cells, phycoerythrin (PE)-conjugated PBS-57 loaded on mouse CD1d tetramers (mCD1d/PBS-57) was obtained from the Tetramer Core Facility of the National Institute of Health.

Measurement of in vivo cell proliferation and ex vivo survival

Mice were given one intraperitoneal injection of 0.2 mg EdU (BD Bioscience). Twelve hours after EdU injection, the mice were sacrificed, and the organs were harvested. Cells derived from the thymus were stained with the antibodies specific for the cell surface antigens described above. After fixation and permeabilization, cells were stained with FITC anti-EdU antibody and 7-AAD (BD Pharmingen) according to the manufacturer's instructions. EdU incorporation into different cell populations was measured by flow cytometry.

For the measurement of cell viability, we stained the thymocyte suspensions with 7-AAD and antibodies against annexin-V (BD Biosciences) and other surface markers, such as CD4, CD8, CD69, TCR β , CD25, and CD44, either ex vivo or 24, 48, or 72 h after in vitro culture in complete medium.

Cell sorting and RNA preparation

Lymphocytes were obtained from the thymus. Immune cell populations, including DN1-4, DP, SP CD4⁺ and CD8⁺, and iNKT cells, were stained in combination with antibodies against the cell-specific markers CD4, CD8, CD69, TCR β , CD25, CD44, and mCD1d/PBS-57 and were subsequently sorted into different subsets using a FACS Aria Cell Sorter (Becton-Dickinson, San Jose, USA). The purity of the sorted cell populations was over 98%, as validated by flow cytometry. The sorted cells were lysed using

TRIzol reagent (Invitrogen) or RLT buffer from the RNeasy Micro kit (Qiagen), and RNA was extracted according to the manufacturer's instructions.

Quantitative RT-PCR

We used a high capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, USA) or iScript cDNA synthesis kit (Bio-Rad) to generate cDNA for RT-PCR. PCR was carried out with a SYBR Green-based kit (FastStart Universal SYBR Green Master, Roche, Basel, Switzerland) or SensiFast SYBR No-ROX kit (Bioline) on a StepOne plus instrument (Applied Biosystems, Carlsbad, USA) or a LightCycler 480 system (Roche). Primers were designed using software from Roche. We used the following primers for mouse QPCR: Zeb1 forward primer, 5'-GCCAGCAGTCATGATGAAAA-3'; Zeb1 reverse primer, 5'-TATCACAATACGGGCAGGTG-3'; Zeb2 forward primer, 5'-CCAGAGGAAACAAGGATTCAG-3'; Zeb2 reverse primer, 5'-AGCCTGACATG

TAGTCTTG-3'; Gapdh forward primer, 5'-GCATGGCCTCCGTGTTTC-3'; Gapdh reverse primer, 5'-TGTCATCACTTGGCAGGTTTC-3'. The relative expression of Zeb1 and Zeb2 were normalized to Gapdh expression.

Western blotting

Cells were lysed in NP40 lysis buffer (20 mM Tris, HCl pH 7.4; 150 mM NaCl; 2 mM EDTA; 1% NP40) containing protease inhibitors for 30 min on ice. The supernatant was collected following 10 min of centrifugation at 12,000 g at 4 °C, and the protein concentration was quantified by a μ BCA quantification kit (Thermo Fisher Scientific). Fifty micrograms of total cellular protein from the thymus was incubated for 5 min at 95 °C. Protein samples were separated by electrophoresis using Novex 4–12% Tris-Glycine gels (Life Technologies) for 1 h at 120 V. The proteins were then transferred to a PVDF membrane (Bio-Rad). After blocking with PBS containing 0.1% Tween and 5% milk for 1 h, the membranes were probed with the following primary antibodies: anti-Gapdh (Cell Signaling Technology, 2118) and anti-Zeb1 (Cell Signaling Technology, 3396; the antibody was raised against a peptide with Asp846, the *Cellophane* mutation truncating the protein after Tyr902) overnight at 4 °C. Membranes were washed three times with PBS containing 0.1% Tween, and secondary antibodies were added for incubation for one hour at RT. Anti-rabbit and anti-mouse HRP conjugate secondary antibodies were provided by Jackson ImmunoResearch. Proteins were revealed with a Chemiluminescence Western Lightening Plus kit (Perkin-Elmer).

RNA-seq analysis

Thymic suspensions were stained in combination with anti-CD3, anti-CD4, anti-CD8, anti-CD69, and anti-TCR β and subsequently sorted into different subsets using a FACS Aria Cell Sorter (Becton-Dickinson, San Jose, USA). The purity of the sorted cell populations was over 98%, as measured by flow cytometry. RNA libraries were prepared as previously described.⁶⁰ Briefly, total RNA was purified from 5×10^4 sorted thymocytes using the Direct-Zol RNA microprep kit (Zymo Research) according to the manufacturer's instructions and was quantified using the QuantiFluor RNA system (Promega). One microliter of 10 μ M oligo-dT primer and 1 μ l of 10 mM dNTP mix were added to 0.15 ng of total RNA in a final volume of 2.3 μ l. Oligo-dT cells were hybridized for 3 min at 72 °C, and reverse transcription (11 cycles) was performed. PCR preamplification was then conducted using 16 cycles. The cDNA was purified with Ampure XP beads (Beckman Coulter), and the cDNA quality was checked with D5000 screen tape and analyzed on a Tape Station 4200 (Agilent). Three nanograms of cDNA was tagged using a NextEra XT DNA sample preparation kit (Illumina). The tagged fragments were further amplified and purified with Ampure XP beads (Beckman Coulter). The tagged library quality was checked with D1000 screen tape and analyzed

on a Tape Station 4200 (Agilent). Sequencing was performed with the GenomEast platform by a member of the “France Génomique” consortium (ANR-10-INBS-0009) on an Illumina HiSeq 4000 sequencing instrument (read length of 1×50 nt).

Measurements of TCR signaling

Calcium response. WT and *Cellophane* thymocytes were first barcoded with anti-CD45 coupled with different fluorochromes and then stained at RT with fluorescent anti-CD4, anti-CD8, anti-CD69, anti-TCR β , anti-CD25, and anti-CD44 antibodies, followed by staining with Indo-1 (1 μ M, Life Technologies) at a concentration of 1×10⁷ cells/ml for 30 min at 37 °C. Following two washes at 4 °C, the cells were resuspended in RPMI medium (0.2% BSA and 25 mM HEPES) and were incubated at 37 °C for 5–10 min prior to acquisition. The samples were acquired on an LSRII (BD) as follows: 15 s of baseline acquisition, addition of anti-CD3 biotin (2C11, 10 μ g/ml), acquisition for 1 min 30 s, addition of streptavidin (Life Technologies, 10 μ g/ml), and acquisition for another 3–5 min.

Phosphorylation events. Different samples corresponding to different mice were barcoded by labeling them with a series of anti-CD45 antibodies coupled with different fluorochromes. For phospho-flow staining, 3×10⁶ mixed thymocytes were stained using biotinylated CD3 (2C11, 5 μ g/ml) and other surface markers for 15 min, followed by streptavidin (Life Technologies, 10 μ g/ml) stimulation and fixation by the addition of 10 volumes of Lyse/Fix at the indicated time points. The levels of pErk, pS6, or pAkt were normalized according to the MFI, which was detected in the nonstimulated condition (regarded as 100%) for each mouse.

In silico analyses

The functional annotations of DEGs were performed using Metascape³⁷ or STRING³⁸ using the default parameters. In addition, we used several functionalities of the ImmGen database browser²⁹ to generate some of the figures included in the supplementary information.

Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad Software). Two-tailed unpaired *t*-tests, paired *t*-tests, and ANOVA with Bonferroni correction were used as indicated. We used the hypergeometric test and the Benjamini–Hochberg *p* value correction algorithm to calculate if the enrichment of the overlap between the gene lists was statistically significant.

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AUTHOR CONTRIBUTIONS

JZ, AB, MW, DL, DEC, ALM, AR, and AM performed the experiments. RS and QM performed the in silico analyses. JC, LG, and YGH provided reagents and conceptual insight and helped write the paper. TW wrote the paper and supervised the work.

ADDITIONAL INFORMATION

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Figure S1

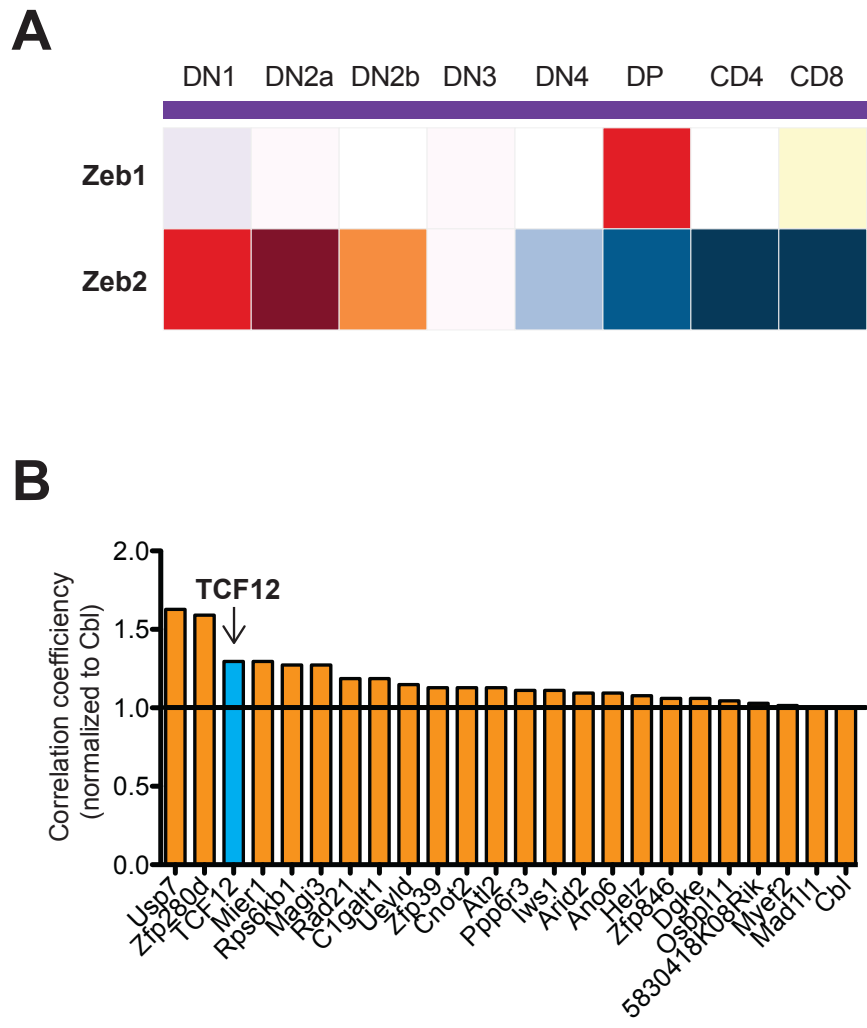


Figure S2

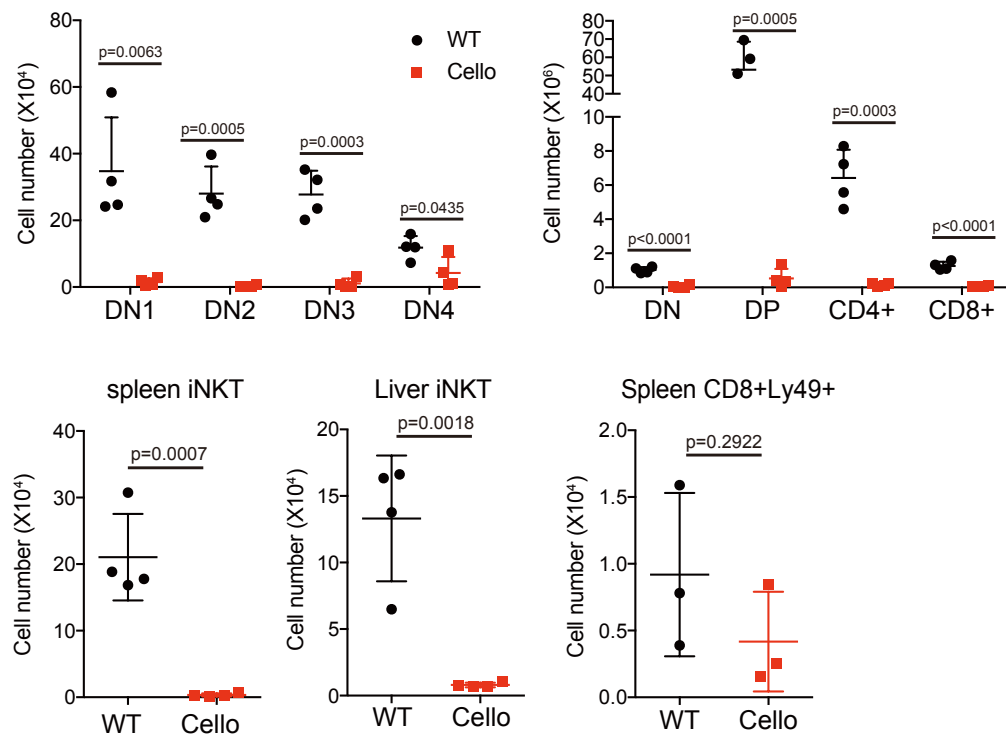


Figure S3

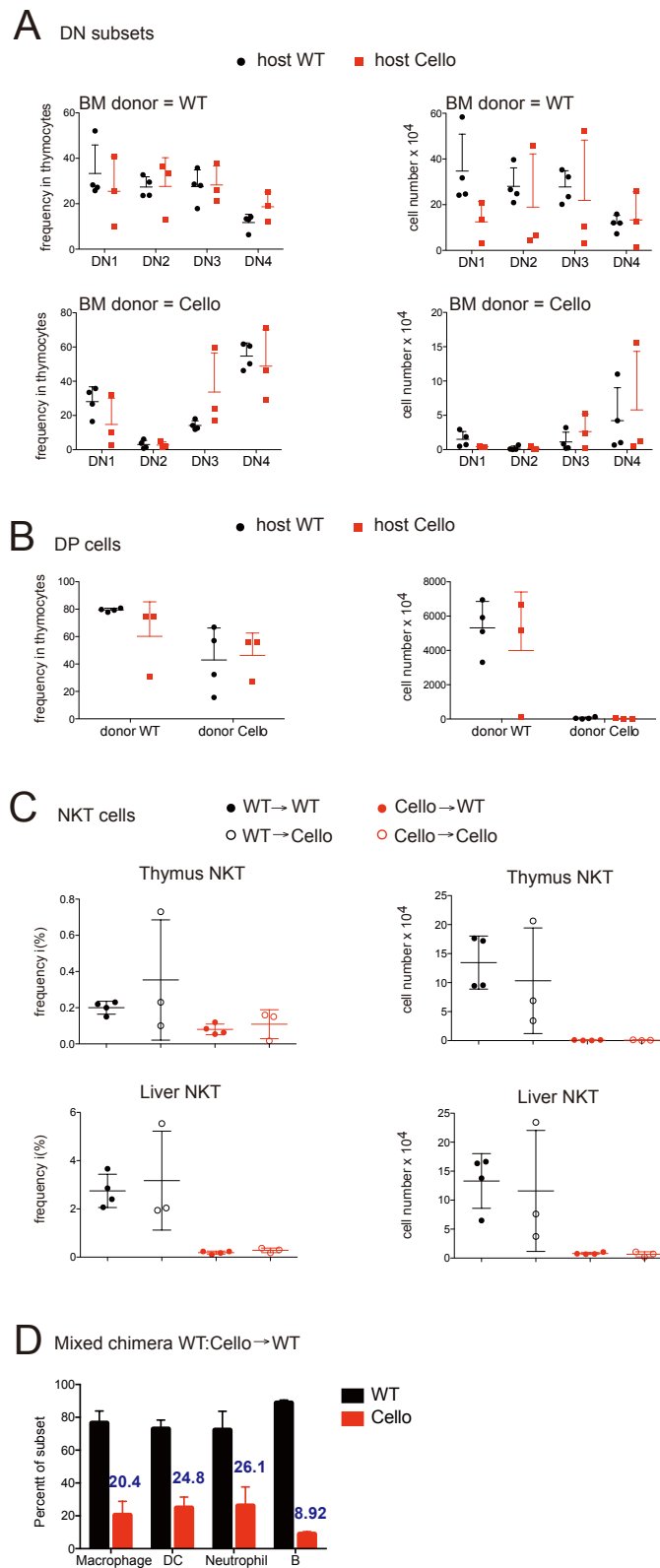
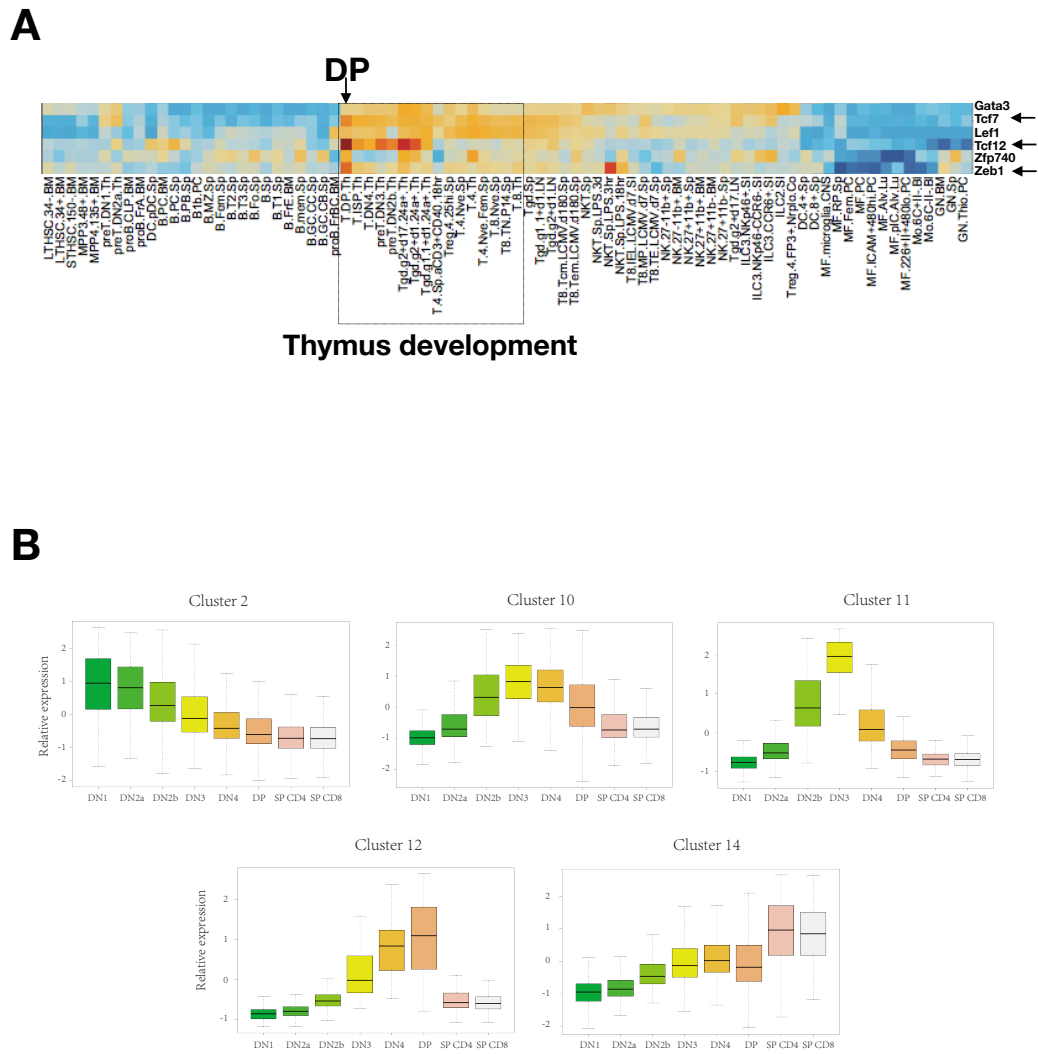


Figure S4



Supplementary figure legends

Figure S1: Zeb1 is highly expressed in DP and co-regulated with Tcf12. (A) Expression profile of Zeb1 and Zeb2 mRNA level in thymic $\alpha\beta$ T cell subsets. The image is adapted from Immgen (<http://www.immgen.org/>). The red color indicates higher expression. (B) Genes for which the expression pattern in $\alpha\beta$ T cell subsets is highly correlated with that of Zeb1. The figure is adapted from data on the Immgen browser (<http://www.immgen.org/>).

Figure S2: Decreased numbers of thymocyte subsets and peripheral NKT cells in *Cellophane* X Ly5a BM chimeric mice. Cell numbers calculated for the panels A-C in Figure 4, as indicated.

Figure S3: The role of Zeb1 in T cell development is cell-intrinsic. (A-C) Flow cytometry analysis of indicated thymocyte subsets or thymus/liver iNKT cells from BM chimeras. Recipients were C57BL/6 (WT) mice or *Cellophane*, as indicated, and donor BM was either Ly5a x C57BL/6 (WT) or *Cellophane*, as indicated. Graphs show the mean \pm SD frequency or number of 3-4 mice in 2 experiments. (D) Mixed BM chimeric mice described in Figure 4D were analyzed by flow cytometry for the indicated subsets. Results show the BM donor origin 983 in percentage for each gated subset. Mean \pm SD of 6 mice.

Figure S4: Zeb1 regulates epigenetic remodeling during T cell development. (A) Snapshot of Figure 5 in Yoshida et al [41], highlighting the cluster of TFs predicted to regulate chromatin accessibility during T cell development. (B) Boxplot graphs showing the mean accessibility of different open chromatin regions (OCRs), as determined by ATAC-seq by Yoshida et al., in developing T cell subsets, as indicated. All OCRs contain Zeb1 binding sites.

4.3.2 Extended results

De-repression of Zeb1 alters T cell development and decreases TCR signaling

A previous article reported that Myb was a repressor of Zeb1 and that this repression was important for inner ear development. This observation was based on the analysis of a mutant mouse called *Twirler* in which a heterozygous noncoding point mutation in the first Zeb1 intron abrogated a Myb binding site and resulted in increased Zeb1 expression (Kurima et al., 2011). The name "Twirler" relates to the mice propensity to revolve in the cage (because of the inner ear problem). Since *Twirler* mice appeared somewhat complementary to *Cellophane* mice for the study of Zeb1, and since Myb is a known regulator of iNKT cell development (Hu et al., 2010), we wanted to analyze T cell development in *Twirler* mice. However, these mice were no longer available, which led us to generate a novel mutant mouse strain in which we deleted 13 nucleotides in the same Zeb1 intronic region using Crispr/Cas9 genome edition. In these heterozygous mice, called $Zeb1^{\Delta 13/+}$, the analysis of thymus composition showed a profound decrease in DN and DP thymocytes, which accounted for the decreased cellularity of this organ (Figure E2A). A Q-RTPCR analysis showed an increased Zeb1 expression in DP of $Zeb1^{\Delta 13/+}$ mice (Figure E2B), confirming the role of Myb as a repressor of Zeb1 expression. $Zeb1^{\Delta 13/+}$ DP displayed accelerated cell death compared to their WT counterpart upon *in vitro* culture (Figure E2C). Moreover, $Zeb1^{\Delta 13/+}$ DP thymocytes showed increased proliferation compared to control DP, as assessed by EdU incorporation or Ki67 measurement (Figure E2D-E), which is the opposite phenotype of *Cellophane* DP. A flow cytometry analysis of DP thymocytes showed a decreased Nur77 and CD5 expression in $Zeb1^{\Delta 13/+}$ DP compared to control DP, suggesting a decreased TCR signaling in mutant DP (Figure E2F). We assessed TCR signaling by stimulating DP through the TCR and measuring calcium flux. We failed to detect reproducible differences between $Zeb1^{\Delta 13/+}$ and control DP, but we noted that $Zeb1^{\Delta 13/+}$ CD4 SP had a decreased calcium response compared to control SP CD4 (Figure E2G-H).

We then analyzed the composition of the peripheral T cell compartment. iNKT cells tended to increase in percentages in the thymus but not in the spleen or liver, and

they were reduced in numbers in all organs analyzed (Figure E2I). NK1.1⁺ $\gamma\delta$ T cells tended to increase in frequency in spleen and LN but their overall numbers were either not changed or even decreased (Figure E2J). Ly49⁺ CD8 T cells were also reduced in number and their frequency was decreased in peripheral organs (Figure E2K). When analyzing iNKT subsets, we observed the opposite phenotype as that in *Cellophane* mice: i.e. an increase of the iNKT2 and iNKT17 subsets at the expense of iNKT1 (Figure E2L), similar to the phenotype of SKG mice, which supported a decreased TCR signaling in *Zeb1* ^{Δ 13/+} thymocytes.

Collectively, these data show that the gain-of-expression *Zeb1* ^{Δ 13/+} heterozygous mutation has profound consequences on T cell development associated with hints of decreased TCR signaling and increased cell proliferation in DP thymocytes, as opposed to increased TCR signaling and decreased cell proliferation in *Cellophane* mutant mice.

***Cellophane* mice show macroscopic organ defects**

As described before, *Cellophane* mice have much smaller thymi than C57BL/6 control mice. Interestingly, the contrary was observed for *Cellophane* spleen, which was usually bigger than that of WT mice (Figure E3A). In order to better understand the causes of splenomegaly, spleen sections were prepared and stained either with hematoxylin phloxine saffron (HPS) or with antibodies directed against CD3 or B220 (Figure E3B). Splens from *Cellophane* had a defective anatomical structure. First, no red pulp could be easily delineated, and the white pulp was completely disorganized. Only few T cells were observable and were disseminated along the organ instead of being organized in clusters, while B cells were observable in important proportion and not assembled in follicles. This defect might be due to "hypersplenism", a condition that can have multiple causes and associated with exaggerated scavenger function resulting in both red blood and white blood cell cytopenia.

Moreover, we found that the spleen from *Cellophane* was difficult to mash indicating splenic fibrosis. Several studies have found a correlation between fibrosis and ILC2 with an involvement of cytokines e.g. IL-25, IL-33 ([Hams et al., 2014](#); [Mchedlidze](#)

et al., 2014). A flow cytometry analysis of spleen cell suspensions showed that ILC1, ILC2, ILC3 cell were all increased in *Cellophane* in terms of cell numbers and percentages while NK cells were oppositely decreased (Figure E3C). To test if Zeb1 had an intrinsic role in ILC2 cell development, we generated bone marrow (BM) chimeric mice by reconstituting sublethally-irradiated Ly5a (CD45.1) with BM from *Cellophane* (CD45.2) and WT Ly5a x C57BL/6 (CD45.1/2) mice. In the spleen of chimeric mice, the frequency of ILC2 cells was mildly lower for *Cellophane*-derived compared to WT (Figure E3D) indicating that Zeb1 might not regulate ILC2 cell development intrinsically.

Zeb1 expression is associated with lymphoma

Zeb1 is best known for its role in EMT through repression of epithelial genes during embryonic development (Vandewalle et al., 2005). Zeb1 also influences tumor progression and metastasis as depletion of Zeb1 suppresses stemness, colonization capacity (Krebs et al., 2017; Spaderna et al., 2008). We sought to detect ZEB1 expression level by western blotting in several T cell lymphoma lines. Results showed that 5 out of 8 cell lines had low levels of ZEB1 expression (Figure E4), suggesting a universal downregulation of ZEB1 in lymphoma. This attracted our attention to further investigate the role of ZEB1 in lymphoma development.

Extended Methods

Generation of Zeb1^{Δ13/+} mice

We used CRISPR/Cas9 genome editing in mice, as previously reported (Teixeira et al., 2018). Briefly, we electroporated intact mouse zygotes (B6D1F1xFVB background) with ribonucleoprotein complexes prepared *in vitro* from recombinant Cas9 nuclease and synthetic dual guide RNA. We designed a guide RNA using the online CRISPOR freeware (<http://crispor.tefor.n-et/>) (Haeussler et al., 2016) optimized to maximize cutting specificity and efficiency. The guide was selected to induce a cut in the region of the Myb binding site of Zeb1, as previously defined (Kurima et al., 2011). The sequence of the guide we used is 5'-TGCCGCTGCCGCCGCCGCTGCCGCCGCCGCCGCCGCCGCGGCTGCTGGACTGGACCATTATGTCTTACCTGCCTCTGCGC-

CTAGCGGCTCCGGCGGCCGCGGGG-3'. Founder mice were genotyped by PCR and sequencing, using primers flanking the target sequence of the guide RNA. Founder mice with the *Zeb1*^{Δ13/+} mutation were backcrossed 3 to 6 times on the C57BL/6 background before use. All control mice were littermates.

Histology and histoimmunochmeistry

Spleens were carefully dissected and kept in a 4% paraformaldehyde solution, before paraffine embedding and microtome slicing. Organs slices were further stained with hematoxilin phloxine saffron (HPS) or using antibodies directed against CD3 (1:100) or B220 (1:100). Slides were observed and pictures were taken using an upright Zeiss Axiolmager Z1 microscope. Images were analyzed using the ImageJ software.

Flow cytometry

Detailed methods were described in the Article 2. Here are the lists of additional antibodies used for ILC subsets gating: anti-mouse Rorγt (clone AFKJ S-9), anti-mouse Gata3 (clone TWAJ), anti-mouse CD127 (clone AFR34), anti-mouse CD90.2 (clone 53-2.1).

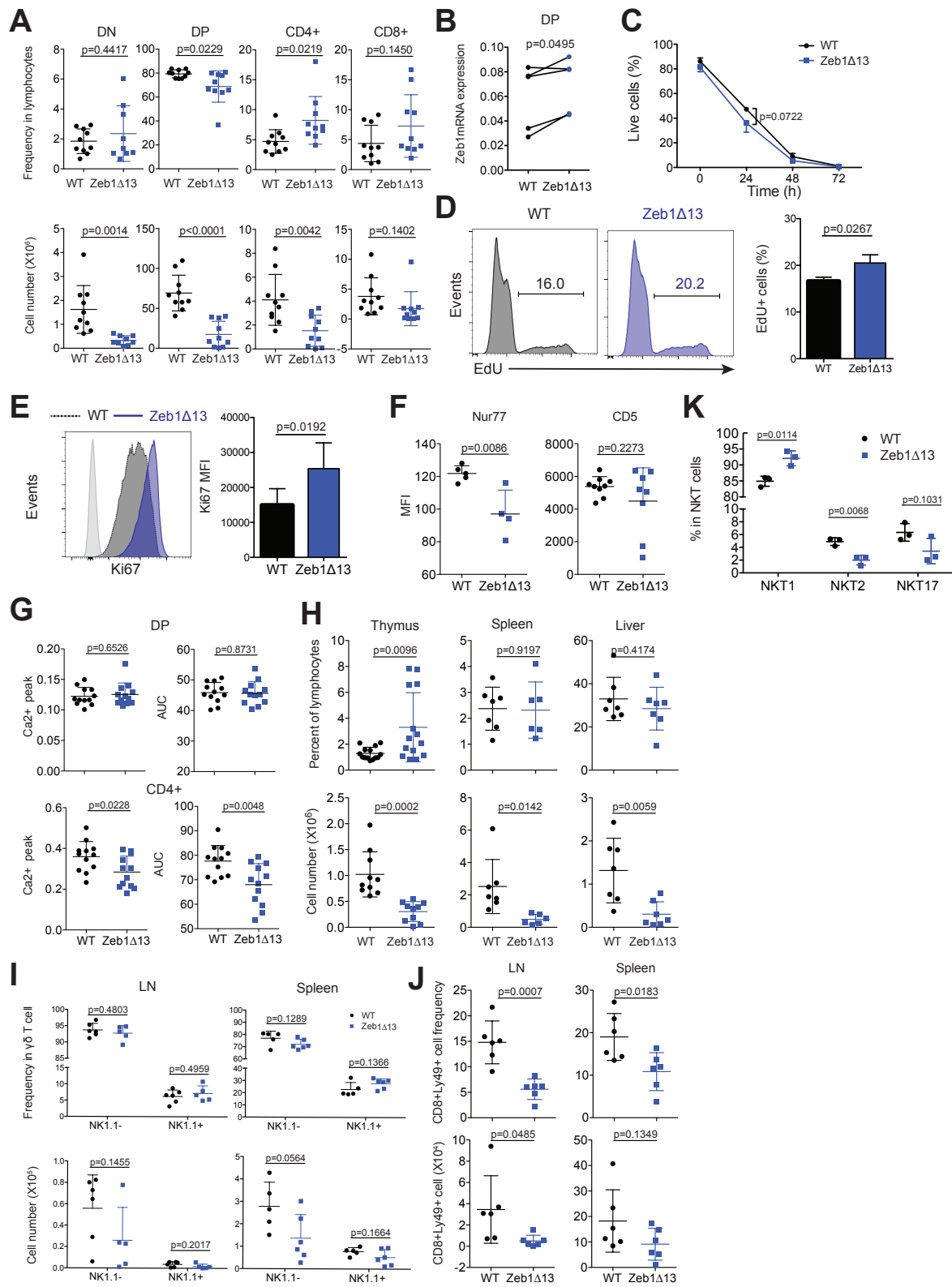


Figure E2: *Zeb1*^{Δ13/+} mutant mice have increased Zeb1 expression and display signs of decreased TCR signaling. (A) Percentage and absolute numbers of thymocyte subsets (CD4 SP, CD8 SP, DN, and DP) in the thymus of wild-type and *Zeb1*^{Δ13/+} mice. N=9 mice in four individual experiments. (B) Q-RTPCR measurement of Zeb1 mRNA expression in sorted DP thymocytes from wild-type and *Zeb1*^{Δ13/+} mice. N=5 for each genotype. (C) Annexin V expression by wild-type or *Zeb1*^{Δ13/+} thymocytes cultured for 0-72h. N=3 mice in each group in 2 independent experiments. (D) EdU incorporation of wild-type and *Zeb1*^{Δ13/+} DP thymocytes after a 12-hour *in vivo* pulse of EdU. N=3 for each genotype. (E) Flow cytometry analysis of Ki67 expression in DP thymocytes from WT and *Zeb1*^{Δ13/+} mice. N=3 for each genotype. (F) Flow cytometry analysis of Nur77, CD5 expression in DP thymocytes of wild-type and *Zeb1*^{Δ13/+} mice. Data are shown as mean fluorescence intensity. N=5-8 for each genotype. (G) Thymocytes were activated following incubation with biotinylated anti-CD3 followed by cross-linking with streptavidin. Dot plots showing the AUC (Area under curve) and Ca²⁺ peak for N=12 mice in four individual experiments. (H) Frequency and number of CD1d-tet⁺ iNKT cells from thymus, spleen, and liver in WT and *Zeb1*^{Δ13/+} mice. N=3-4 in 2 experiments. (I-J) Absolute number and frequency of NK1.1⁻ or NK1.1⁺ γδ T cells within total γδ T cells (I) and Ly49⁺ cells within CD8⁺CD44⁺ T cells (J) for LN and spleen of WT and *Zeb1*^{Δ13/+} mice. N=5-6 mice in 2 experiments. Statistical analysis was performed using unpaired Student *t* test.

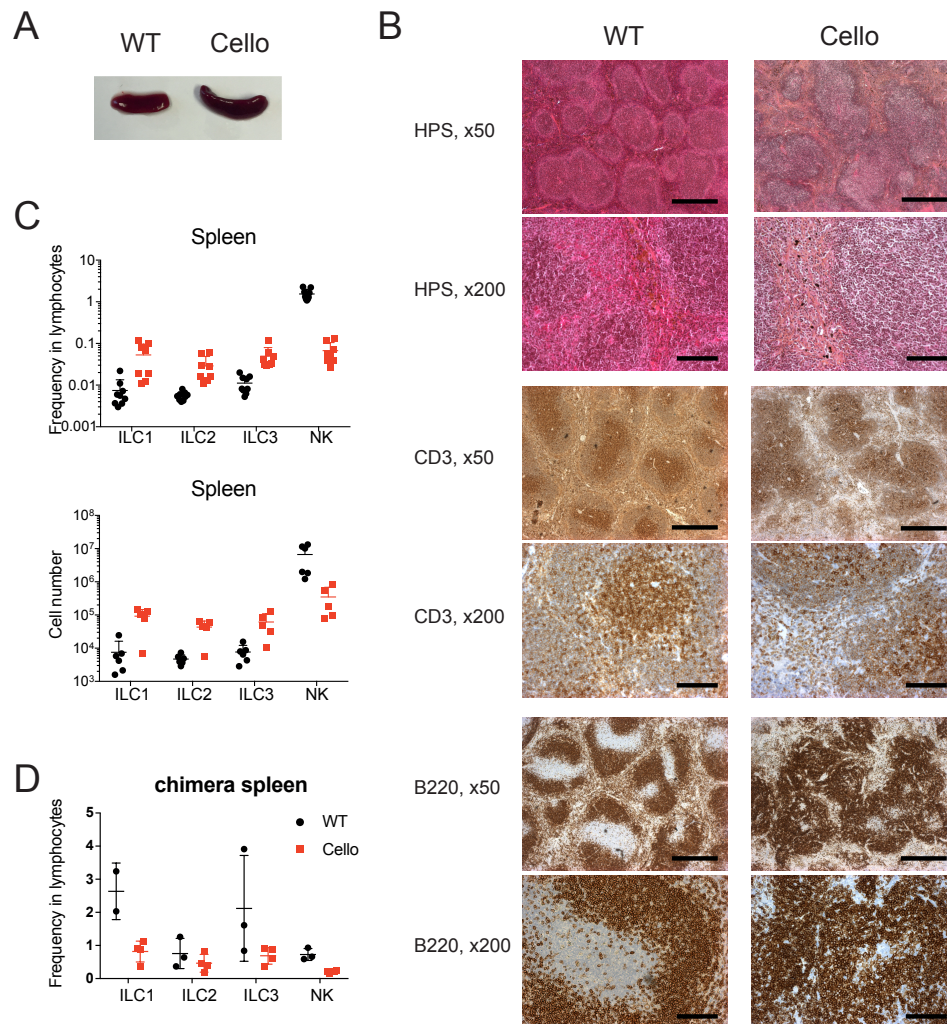


Figure E3. *Cellophane* mice show macroscopic organs defects. (A) Spleen of both *cellophane* and WT mice. The scale represents 1 cm. (B) Microscopic observations of spleens of both old WT and *cellophane* mice. Haematoxylin phloxine saffron stain was performed, as well as immunohistochemistry with antibodies directed against either CD3 or B220. Scale bars represent 500 μ m for the x50 magnification and 100 μ m for the x200 magnification. (C) Frequencies and numbers of cells in the thymus. Error bars represent standard error to the mean. (D) Frequencies of cell in chimera mice with a mix of BM cells from WT and *Cellophane* mice.

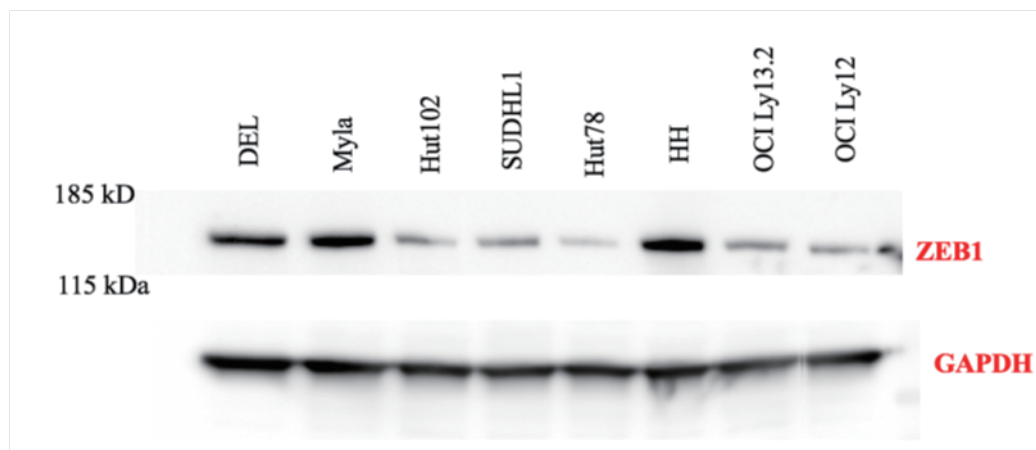


Figure E4. Most of lymphoma cell lines show decreased expression of ZEB1. WB analysis of ZEB1 expression in total lysat of different lymphoma cell lines .

5 Discussion

5.1 Project 1: T-bet and Eomes in NK cell development

In our study, we carefully analyzed the role of T-bet and Eomes in NK cell development at both epigenetic and transcriptomic level. We show that Eomes and T-bet play complementary roles in the control of the NK cell maturation program. Eomes was dominantly expressed in immature NK cells and strongly affected the transcriptome of immature NK cells. Oppositely, T-bet was dominantly expressed in mature NK cells, and was absolutely essential for terminal NK cell maturation. Eomes and T-bet were essential for the $CD11b^- \rightarrow DP$ and $DP \rightarrow CD27^-$ transition, respectively. Although genome-wide analysis of T-bet and Eomes binding indicated strong overlap in DNA-binding properties, Eomes and T-bet have different impact in chromatin accessibility at different stages further demonstrating a labor-division role of T-bet and Eomes during NK cell maturation. Our work raised several important questions, as detailed in the following section.

5.1.1 Eomes and tissue-residency

Among the few transcription factors regulated by Eomes, Hobit turns out to be negatively regulated by Eomes based on the transcriptomic data. Hobit was previously reported to control tissue residency in lymphocytes, in cooperation with Blimp1 ([Mackay et al., 2016](#)). In the liver, mutually exclusive expression of CD49a and CD49b can divide liver NK cells into two distinct cell populations: $CD49a^+CD49b^-$ and $CD49a^-CD49b^+$, considered as tissue-resident NK (trNK) cells (or ILC1s) and conventional NK (cNK) cells ([Peng and Tian, 2017](#)). trNK cells have much higher level of Hobit than cNK cells at the mRNA level ([Mackay et al., 2016](#)). Hobit was also shown to be expressed by a subset of human liver-resident $CD56^{bright}$ NK cells ([Lunemann et al., 2017](#)). Moreover, two receptors CD62L and S1PR5 that are essential for blood circulation are also induced by Eomes. We also found that there is an increased proportion of $CD49a^+CD49b^-$ cells in Eomes-deficient liver

compared to the control mice (Figure 24). Collectively, we propose that Eomes may promote blood circulation by inducing CD62L and S1PR5 and by repressing Hobit in NK cells, which is well supported by the different distributions of NK cells and ILC1s, the latter being Eomes negative and tissue-resident (Daussy et al., 2014).

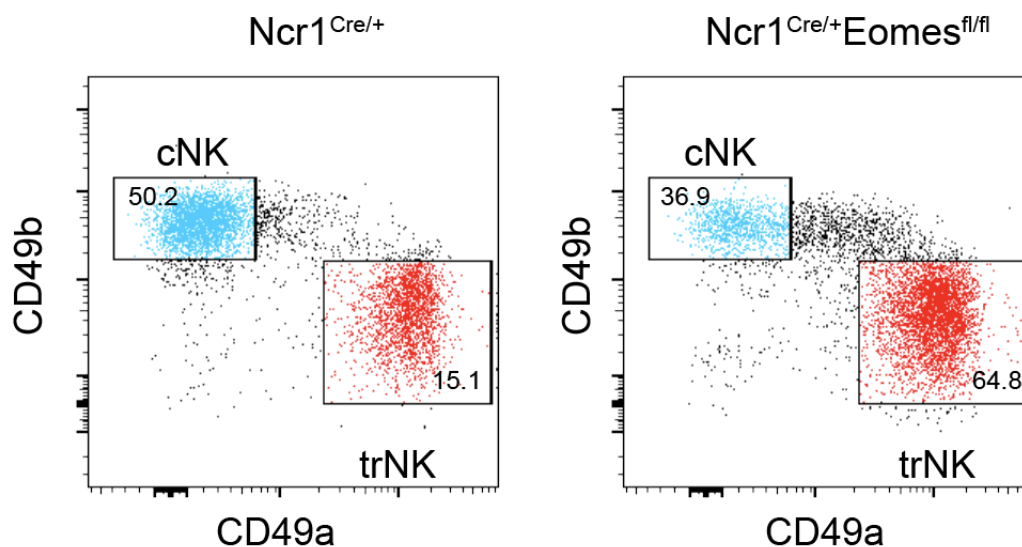


Figure 24. cNK and trNK in the liver. FACS plots of CD49a and CD49b on liver CD3⁻NK1.1⁺ cells are shown within *Ncr1^{Cre/+}* and *Ncr1^{Cre/+} x Eomes^{fl/fl}* mice. cNK cells (CD49a⁻CD49b⁺) decreased in Eomes KO mice while trNK cells (CD49a⁺CD49b⁻) increased almost 3 times compared to the control.

Our data show that Blimp1 and S1PR5 expression are attenuated by T-bet deficiency. In terms of circulation, Blimp1 is a positive regulator of tissue-residency (Mackay et al., 2016) while S1PR5 positively promotes NK cells egressing from BM (Mayol et al., 2011). We found that there were overall more NK cells in the BM of *Tbx21^{-/-}* mice compared to controls and less NK cells in other organs, suggesting that T-bet is essential for the egress of NK cells from BM through S1PR5 although T-bet is lowly expressed in the BM. T-bet is indispensable for ILC1 cell development as almost no ILC1 cells were detected in T-bet KO mice (Sojka et al., 2014; Daussy et al., 2014). And T-bet is important to promote NK cell responsiveness to IL-12 and to support their blood circulation from LN through the upregulation of S1PR5, which could be important for their capacity to control lung metastases in the B16 model (Werneck et al., 2008; van Helden et al., 2015). Besides, Blimp1 is also required for NK cell maturation and homeostasis through IRF4-Blimp1-Bcl6 regulatory axis (Kallies et al., 2011). Therefore, T-bet seems to mainly regulate genes like Blimp1 and S1PR5

mediating both migration and functions.

5.1.2 Eomes, T-bet and CD11b, CD27

Here, we demonstrated that Eomes and T-bet have complementary role during NK cell maturation based on CD11b and CD27 expression (Chiossone et al., 2009). However, the FACS plot shows that the maturation program of NK cells is continuous (Figure 25) and the border between stages is not clear. It makes the sorting of different populations

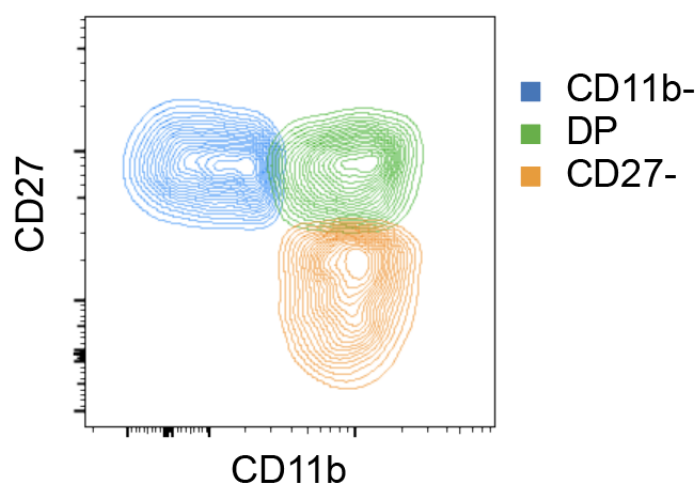


Figure 25. FACS plot on NK cell maturation. Gated on CD3⁻NK1.1⁺CD49a⁻CD49b⁺ cells, NK cells are further divided into three different maturation stages based on CD11b and CD27. The cloud in blue represents the most immature NK cells (CD11b⁻CD27⁺); the cloud in green is the DP intermediate stage (CD11b⁺CD27⁺) and then further mature into stage 3 CD27⁻ (CD27⁻CD11b⁺) (in orange).

tricky and the exclusion of cells inbetween is difficult. Moreover, deficiency of T-bet promotes the expression of CD27 and deletion of Eomes impairs CD11b (encoded by gene *Itgam*) expression. Since we define the maturation of NK cells in terms of CD11b and CD27 level, it might bias the final conclusion. In *Tbx21*^{-/-} mice, there are barely CD27⁻ cells detected suggesting a role of T-bet in the DP→CD27⁻ transition. Since T-bet regulates CD27 itself, it is not valid to make that conclusion simply based on the CD11b and CD27 markers. However, we found that many other molecules (KLRG1, inhibitory receptors) associated with maturation were regulated by T-bet and Eomes indicating an important role of T-bet and Eomes during NK cell maturation. Taken together, in order to get full images of NK cells and to exclude the potential bias

brought by different maturation definitions, transcriptomic and epigenetic modification at the single-cell level or even spacial profiling would be an interesting way to tackle the problems.

5.1.3 T-box family and proliferation

NK cells mirror adaptive CD8⁺ T cells in terms of transcriptional regulation and cytotoxic functions. Particularly, T-bet and Eomes are highly expressed in effector CD8⁺ T cells where the two members of T-box family have been widely investigated. The differentiation of antigen-specific effector CD8⁺ T cells occurs along a continuum as it is in NK cells (Kaech and Cui, 2012). The acquisition of T-bet endows the CD8⁺ T cells with more terminally differentiated phenotypes (KLRG1^{hi}IL-7R α ^{low}) and a reduction in proliferative capacity and longevity (Joshi et al., 2007), while Eomes counter-regulates and also interacts with T-bet thus preventing terminal differentiation of effector T cells and helps to maintain memory cell properties (Intlekofer et al., 2005; Banerjee et al., 2010). In memory CD8⁺ T cells, T-bet and Eomes are reported to cooperatively regulate IL-2R β (also known as CD122) expression which enables IL-15-mediated signaling and homeostatic proliferation (Intlekofer et al., 2005).

CD122 expression was decreased upon deletion of Eomes but increased in the absence of T-bet in NK cells. As CD122 is the rate-limiting factor for the use of IL-15, this could explain why there are so few NK cells in Eomes-KO mice. Our ChIP-seq analysis showed that both T-bet and Eomes can bind to CD122 in the promoter region indicating a competitive role of DNA binding of T-bet and Eomes. Whether the binding is activating or repressive requires further information like additional histone modification. However, we show that deletion of either T-bet or Eomes promotes *in vivo* NK cell proliferation but decreases survival under stimulation of IL-15 *in vitro*.

Furthermore, Bhlhe40 is another transcription factor positively regulated by Eomes based on the RNA-seq analysis. A recent study suggested that Bhlhe40 could be especially important for memory CD8 T cells through the control of bioenergetic metabolism and critical for T cell responsiveness following anti-PD-L1 blockade (Li et al., 2019). Therefore, Eomes may interfere the metabolism of NK cell through

regulating Bhlhe40, as a strong defect of survival in Eomes-deficient NK cells was also detected. How T-bet and Eomes are driving the clonal proliferation of NK cells remains to be elucidated.

5.1.4 T-box family and cytokine signaling

In our study, we demonstrated that upon a 24h-stimulation of IL-12 and IL-18, T-bet levels increased while Eomes levels tended to decrease, opening the possibility that cytokine signals in the microenvironment might determine the balance between Eomes and T-bet. The effect of IL-12 in inducing T-bet expression and repressing Eomes was also reported for effector CD8 T cells in part through inhibition of the transcriptional activator forkhead box O1 (FOXO1) (Takemoto et al., 2006; Joshi et al., 2007). IL-2 stimulation induced Eomes expression but not T-bet in CD8 T cell *in vitro* (Pipkin et al., 2010). Moreover, TGF β signaling resulted in T-bet and Eomes downregulation specifically in tissue-resident memory T cells and, reciprocally, T-box proteins controlled TGF β cytokine signaling (Mackay et al., 2015). Taken together, T-bet and Eomes can be regulated by different cytokines although few studies were performed in NK cells. As different combinations of cytokines might exist in different niches (homeostatic or inflammatory, lymphoid tissue or non-lymphoid tissue), it could explain how distinct patterns of T-bet and Eomes expression may occur. By modulating the balance between Eomes and T-bet, cytokines could therefore change NK cell maturation states, even though this point deserves further investigation.

5.1.5 Working model of T-bet and Eomes actions

T-bet and Eomes are the only T-box TF members expressed in the immune system and belong to the same Tbr1 subfamily based on the conservation of the T-box binding domain. The high identity between T-bet and Eomes in the T-box region results in great similarities in DNA-binding sites as previously reported in activated CD8 T cells (van der Veecken et al., 2019) and which we confirmed through our CHIP-seq analysis on NK cells here. Yet, about one-third of T-bet or

Eomes-associated peaks were specific, according to the statistical analysis that was used during the peak calling bioinformatics procedure. However, upon visual inspection of these specific peaks, the difference in peak height between ChIP-seq tracks was not always obvious. This could be due to the low expression of endogenous TFs and thus more experiments would be needed to formally demonstrate the specificity of T-bet and Eomes binding.

Comparisons between TF binding events and expression profiling data in NK cells, revealed a relatively small overlap between TF occupancy and the expression of neighboring genes, with around 27% for both T-bet and Eomes. Previous studies have demonstrated that this overlap was 50% of TF binding events in yeast ([Gao et al., 2004](#)) and 10-25% in mouse ([Vokes et al., 2008](#)). This suggests that not all TF binding events are indeed functional and the vast majority of individual binding events may merely reflect chromatin accessibility ([Li et al., 2011](#)). Therefore, although some TF binding events do not lead to an immediate response in terms of gene expression, they may have a functional role in chromatin remodeling.

Moreover, our study suggested that T-bet and Eomes binding sites are mostly regarded to be indirect as Runt and Ets motifs are enriched under TF binding peaks. Several lines of evidence have already demonstrated the cooperation between both T-bet and Eomes and Runx3 during CD8 T cell differentiation ([Istaces et al., 2019](#); [Cruz-Guilloty et al., 2009](#)). In NK cells, two studies revealed the potential binding between Runx3 and T-box family based on Runx3 ChIP-seq assay on NK cells ([Lotem et al., 2013](#); [Levanon et al., 2014](#)) suggesting a similar cooperation of T-box proteins and Runx3 in NK cells.

Surprisingly, we found an anti-correlation between T-bet and Eomes protein levels in NK cells. On one hand, Eomes expression dropped during maturation while T-bet rose, and vice versa. On the other hand, the level of each factor decreased in the absence of the other one. Moreover, ectopic expression of T-bet results in a lower Eomes level in NK cells ([Daussy et al., 2014](#)) demonstrating that T-bet and Eomes repress each other's expression. Taken together, these data suggest that both TFs act in a complementary manner as one factor might compensate for the absence of the other during maturation. Another explanation for the "antagonized" effect might be due

to a dose-dependent effect which means that NK cells need to keep at a stable level of total T-box family proteins.

In the context of embryonic development and cell differentiation, many pioneer TFs, which were first identified in yeast (Almer et al., 1986), initiate the assembly of transcriptional complexes or nucleosome repositioning so as to increase accessibility for other factors. Pioneer factors can bind to DNA that is inaccessible to other factors (presumably motifs that are within nucleosomal DNA or where the DNA exits the nucleosome) and can recruit chromatin remodeling complexes that lead to nucleosome repositioning (Spitz and Furlong, 2012).

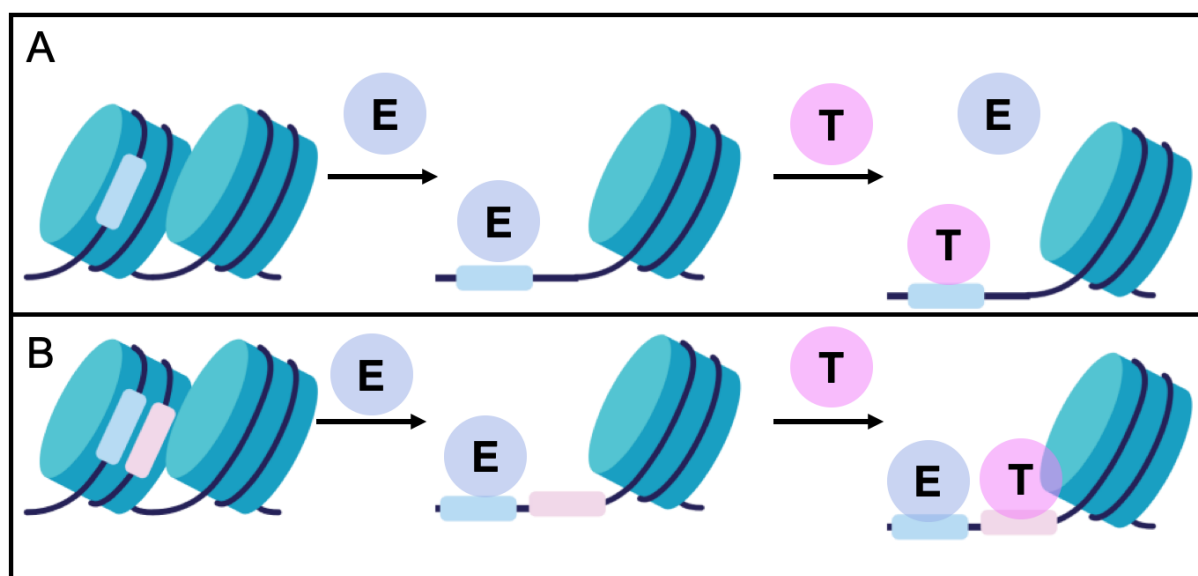


Figure 26. Working model for Eomes and T-bet. Eomes may act as a pioneer factor that opens the chromatin region at early maturation stages. After chromatin remodeling, T-bet either (A) competes with the same binding site as Eomes or (B) binds to a different motif.

In our case, we found that Eomes regulates genes mainly associated with immature signature while T-bet participates in regulating mature signature genes of NK cells both transcriptionally and epigenetically. Eomes is expressed at a high level at immature stage while T-bet expression gradually increases upon terminal maturation. Moreover, more OCRs are detected in mature NK cells (1315) than immature ones (714). Collectively, we propose that Eomes, as a pioneer factor, directly or indirectly (e.g. via Runx3) binds to some inaccessible loci at the immature stage, opens the chromatin region, and facilitates the loading of T-bet either through competition or through co-bound regions (Figure 26). T-bet would then stabilize gene expression —

as is the case for Prf1, GzmA or S1pr5- or in other cases recruit a repressing transcriptional complex (Zeb2, Blimp1, NCOR1 etc) that would switch off transcription — this would be the case for CD27, CXCR3, CD69 and others.

5.2 Project 2: Zeb1 in iNKT cell development

Here, thanks to the use of different transgenic models, we demonstrated an essential role of Zeb1 in regulating T cell development and selection at the DN2 and DP stages. *In vivo* EdU incorporation and *in vitro* survival assay in *Cellophane* mice indicated that Zeb1 is involved in regulating cell proliferation and survival especially at DN2 and DP stages. In addition, Zeb1 dampens TCR signal strength at the DP stage in *Cellophane* mice. Further transcriptomic analysis on DP cells from WT and *Cellophane* mice uncovered a broad array of regulated genes related to TCR signal and cell proliferation. In addition, there are several intriguing points that are worth discussing here.

5.2.1 *Cellophane* and *Zeb1*^{Δ13/+} mice

Zeb1^{Δ13/+} mice have a decreased number of DP cells with increased proliferation, and lower TCR signaling. We failed to detect differences in calcium flux or Akt/S6 phosphorylation between *Zeb1*^{Δ13/+} and control DP upon stimulation. However, we reproducibly found a reduced expression of Nur77 in *Zeb1*^{Δ13/+} DP compared to control DP, evocative of decreased TCR signal strength. This effect could decrease positive selection in T cell development in *Zeb1*^{Δ13/+} mice, which would explain the reduction in DP numbers. We also observed a slight increase in thymic iNKT cells that could be due to the overall decrease in TCR signaling. Yet, we did not observe increased numbers of peripheral iNKT or NK1.1⁺ γδT cells or Ly49⁺ CD8 T cells, as could have been expected from the decreased TCR signaling. Increased Zeb1 expression in mature T cells and in particular innate-like T cells in *Zeb1*^{Δ13/+} mice could therefore also negatively impact their survival. The most robust phenotype in *Zeb1*^{Δ13/+} mice appeared to be the increased proliferation of DP. This demonstrates that a major function of Zeb1 is to sustain proliferation of DP thymocytes. Interfering with this function is obviously expected to have important consequences on the thymic general output. However, we did note a decreased calcium response of *Zeb1*^{Δ13/+} CD4⁺ SP thymocytes compared to control SP, and the distribution of thymic iNKT subsets from *Zeb1*^{Δ13/+} mice was consistent with decreased TCR

signaling. Thus, several lines of evidence indicated a decreased TCR signaling in developing T cells from *Zeb1*^{Δ13/+} mice as a consequence of increased Zeb1 expression.

Overall, the two Zeb1 mutant mouse models advanced our understanding on the function of Zeb1. In terms of expression, Zeb1 is decreased in *Cellophane* and increased in *Zeb1*^{Δ13/+} mice indicating an opposite effect on these two transgenic mice. However, we should keep in mind that the Zeb1 mutation sites in *Cellophane* and *Zeb1*^{Δ13/+} mice are different. The point-mutation in the C-terminal zinc finger domain of *Zeb1* gene in *Cellophane* creates a pre-stop codon resulting in a truncated form of Zeb1 lack of C-terminal zinc finger, while *Zeb1*^{Δ13/+} mice harbor a 13bp-deletion in the first intron which is supposed to be a Myb binding site. What was observed in *Cellophane* mice is likely the consequence of the truncation of the C-terminal ZF domain, while the defects shown in *Zeb1*^{Δ13/+} mice might be more related to disturbed transcriptional regulation. c-Myb is expressed throughout T cell development in the thymus and Myb mRNA expression is upregulated as thymocytes differentiate from the double-negative into the metabolically quiescent, small, preselection DP stage during T cell development (Yuan et al., 2010). Using a conditional knockout model, Myb was demonstrated to be essential for T cell differentiation including 1) transition through the double-negative 3 stage, 2) survival of preselection CD4⁺CD8⁺ thymocytes, and 3) differentiation of CD4 thymocytes (Bender et al., 2004). Another study showed that Myb was also involved in priming CD4⁺CD8⁺ DP cells for selection into the iNKT lineage (Hu et al., 2010). Intriguingly, Myb-deficient DP thymocytes undergo premature apoptosis in an αβ TCR-independent manner with decreased Bcl-xL expression (Yuan et al., 2010). Therefore, it would be interesting to analyze if the decrease of DP population found in *Zeb1*^{Δ13/+} mice is also related to increased apoptosis through Bcl-xL.

However, Myb-KO mice do not phenocopy *Zeb1*^{Δ13/+} mice. In Myb KO mice, a strong decrease of NKT cells was reported both in terms of frequency and number (Hu et al., 2010). In *Zeb1*^{Δ13/+} mice the number of iNKT cells was decreased but their frequency rather increased in the thymus. Therefore, the association between Zeb1 and Myb deserves further investigation and Myb likely has Zeb1-independent effects

that contribute to T cell development. Additionally, the *Zeb1*^{Δ13/+} mice that were used here were all heterozygotes as no homozygous offspring was obtained suggesting a stronger effect in *Zeb1*^{Δ13/+} mice than in *Cellophane* mice. Moreover, since we only backcrossed 3-6 times on the C57BL/6 background before use, the difference between mouse strains might complicate the interpretation. More experiments are thus required using *Zeb1*^{Δ13/+} mice with a cleaner background.

5.2.2 Splenomegaly, fibrosis and ILCs

Despite their defect in T cell development, *Cellophane* mice have an enlarged spleen with disordered red pulp and white pulp compared to the control which might be due to "hypersplenism". "Hypersplenism" is a common disorder characterized by an enlarged spleen, which causes rapid and premature destruction of blood cells (Lv et al., 2016). Although the physiopathology of hypersplenism is still poorly understood, several mechanisms might be involved such as enhanced macrophage phagocytosis, upregulation of cytokines or autoimmunity (Lv et al., 2016). Malfunction of B cells due to dysregulation of cytokine profiles is also associated with "hypersplenism" (Charrier et al., 2013). *Cellophane* mice were first reported to have humoral defects via both T-dependent and T-independent pathways. B-cell development in the bone marrow of *Cellophane* was normal in that the frequencies of pro-/pre-, immature, and mature B cells were equivalent between age- and sex-matched WT and mutant mice. However, *Cellophane* mice lacked discrete populations of MZ B cells in the spleen and had severely reduced frequencies of peritoneal B1 B cells (Arnold et al., 2012). And in our microscopic examination, B cells were found disorganized in spleen as well. Whether B cells are essential for the splenomegaly requires formal demonstration.

Spleen stiffness — supposed to be due to fibrosis — is another intriguing phenomenon. Although the pulmonary fibrosis has been widely investigated, the mechanism of splenic fibrosis remains largely unknown. Emerging evidence showed that ILCs and unconventional T cells contribute to a variety of chronic lung diseases including pulmonary fibrosis, allergic airway inflammation, and chronic obstructive

pulmonary disease (COPD) (Borger et al., 2019; Ardain et al., 2019). Epithelial cell-derived mediators are critical in the regulation of ILC responses. For example, IL-33 has been shown overexpressed in lungs of patients with pulmonary fibrosis and was supposed to be a main driver for ILC2 expansion (Lee et al., 2017). Moreover, ILC2-deficient mice had increased IL-33 expression providing evidence of an essential role for ILC2s in airway fibrosis and lung remodeling processes (Donovan et al., 2019). ILC3s can secrete fibroblast activating cytokines like IL-17 and elevated levels of IL-17 have been observed in patients with cystic and pulmonary fibrosis (Saleh et al., 2009; Brodlie et al., 2011). Therefore, the mechanisms underlying fibrosis could be a combination of multiple immune defects. In our study, we initially found that *Cellophane* mice have an increased number of ILC subsets in the spleen, which might be the cause of fibrosis which could in turn promotes the ILC2 expansion. However, in BM chimera mice we did not observe the same effect in terms of ILC2 frequency in the spleen suggesting a cell-extrinsic effect of the *Cellophane* mutation. Together, more studies are required to understand if the truncated form of Zeb1 also abrogates the cytokine profiles of epithelial cells or other immune cells like myeloid cells that could contribute to fibrosis.

Similarly, loss of ZEB2 specifically in the murine adult hematopoietic system also results in BM fibrosis and splenomegaly with enlarged spleens containing a significant infiltration of hematopoietic stem cells and megakaryocyte/erythroid progenitor cells, indicative of extramedullary hematopoiesis that resemble human myeloproliferative disorders and some other models of myeloproliferation disorders particularly GATA1^{low} mice (Wu et al., 2016; Li et al., 2017). Global gene expression and intracellular signal transduction analysis revealed perturbations in specific cytokine and cytokine receptor-related signaling pathways following Zeb2 loss, especially the JAK-STAT and extracellular signal-regulated kinase pathways (Li et al., 2017). Based on this, it would be interesting to study cytokine secretion and progenitor cells in Zeb1 transgenic mice.

Moreover, as a regulator of proliferation, differentiation, apoptosis in various cells, transforming growth factor- β 1 (TGF- β 1) is considered as a crucial mediator of fibrogenesis involved in renal fibrosis, hepatic fibrosis and pulmonary fibrosis (Chen

et al., 2018; Eser and Jänne, 2018; Katz et al., 2016). TGF- β 1 also induces tissue scarring largely by activating its downstream Smad signaling (Figure 27) (Meng et al., 2016; Hu et al., 2018). Smads mostly exert their functions through post-translational modifications such as phosphorylation, acetylation, sumoylation, ubiquitination and protein-protein interactions (Hu et al., 2018). The transcription factor Zeb1 also contains a SMAD-binding domain (SBD) which might contribute to fibrogenesis.

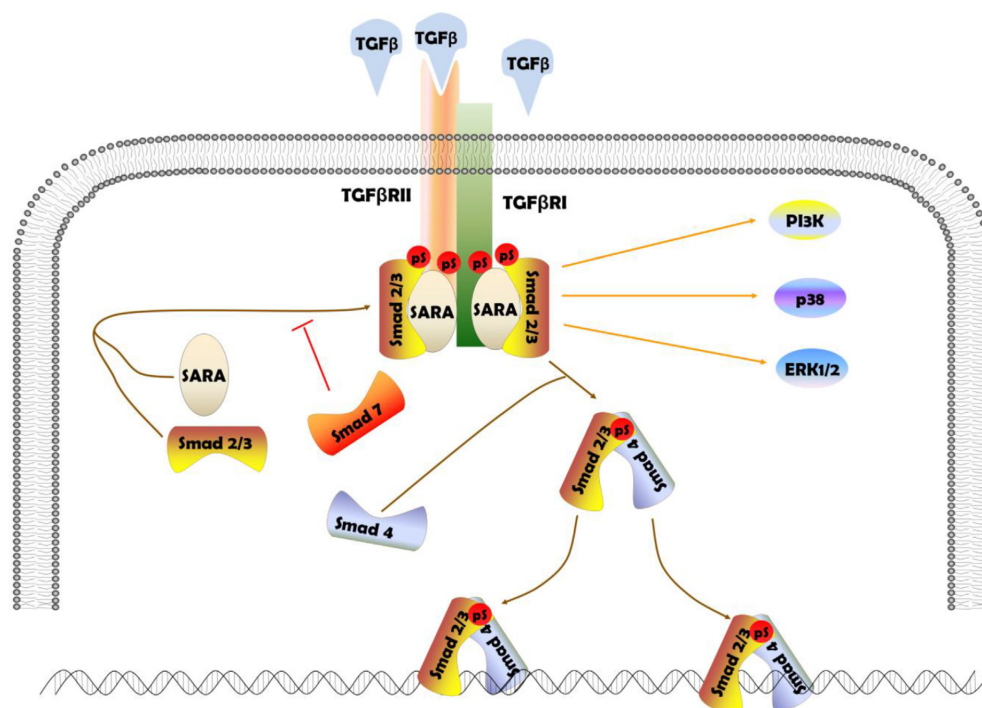


Figure 27. TGF- β /Smad pathway involved in fibrogenesis. Many studies have demonstrated that dysregulation of TGF- β 1/Smad pathway was an important pathogenic mechanism in tissue fibrosis. Smad2 and Smad3 are the two major downstream regulator that promote TGF- β 1-mediated tissue fibrosis, while Smad7 serves as a negative feedback regulator of TGF- β 1/Smad pathway thereby protects against TGF- β 1-mediated fibrosis. Adapted from Hu et al. (2018).

5.2.3 Zeb1 and lymphoma

Zeb1 is best known for its role in epithelial-to-mesenchymal transition (EMT) through the repression of epithelial genes during embryonic development (Vandewalle et al., 2005). Zeb1 also influences tumor progression and metastasis as depletion of Zeb1 suppresses stemness, colonization capacity (Krebs et al., 2017; Spaderna et al., 2008). The Zeb1 genomic region was frequently deleted in cutaneous T cell lymphomas (CTCLs) in a cohort of 43 patients and the survival analysis shows a

worse clinical course for patients with ZEB1 biallelic inactivation, indicating that ZEB1 germline variants might contribute to the risk of developing this disease (Caprini et al., 2018). By further comparing the gene expression patterns of Zeb1-deficient and Zeb1-expressing patients, Zeb1 absence was reported to be associated with the dysregulation of multiple pathways including the reactive oxygen species production and cell viability and apoptosis (Caprini et al., 2018). Moreover, dysregulation of ZEB1 expression was frequently found in adult T-cell leukemia/lymphoma (ATLL) patients compared to healthy people (Figure 28) (Hidaka et al., 2008). Interestingly, a transgenic mouse model lacking Zeb1 COOH-proximal zinc finger clusters (δ EF1), mice were found to develop lymphoma with a median onset of disease of 30 weeks after birth and an earliest onset at 95 days after birth (Hidaka et al., 2008). Half of the mice died within a year, and 84% of them developed fetal T-cell lymphomas. Two types of lymphoma were observed in these mice: (1) periphery lymphomas with or without ascites, and (2) thymic tumors (Hidaka et al., 2008).

In the *Cellophane* colony, we sometimes observed mice with a highly enlarged abdomen due to ascites at old age. It would be interesting to determine if these ascites are caused by an underlying lymphoma. However, not all the phenotypes are consistent between *Cellophane* and δ EF1 mice, e.g. the size of thymus in the *Cellophane* is always smaller than the control while δ EF1 mice had large thymic tumors with a diameter of 1 to 3cm (Hidaka et al., 2008). A recent study showed that Zeb1 expression was induced by TGF- β in conventional CD8 T cells stimulated through the TCR and was essential for memory T cell survival and function (Guan et al., 2018). TGF- β 1 deficient mice showed a defect in the selection and maturation of thymocytes with a reduction in DP and an increase in DN and CD4⁺ in older knock-out mice (Licona-Limon and Soldevila, 2007). ATLL cells were resistant to growth inhibition by TGF- β 1 even when treated with high levels of TGF- β 1 (Kim et al., 1990; Arnulf et al., 2002). Although we failed to detect any effect of recombinant TGF- β on Zeb1 expression in thymocytes (data not shown), it would be interesting to further investigate the link between Zeb1, TGF- β and tumorigenesis.

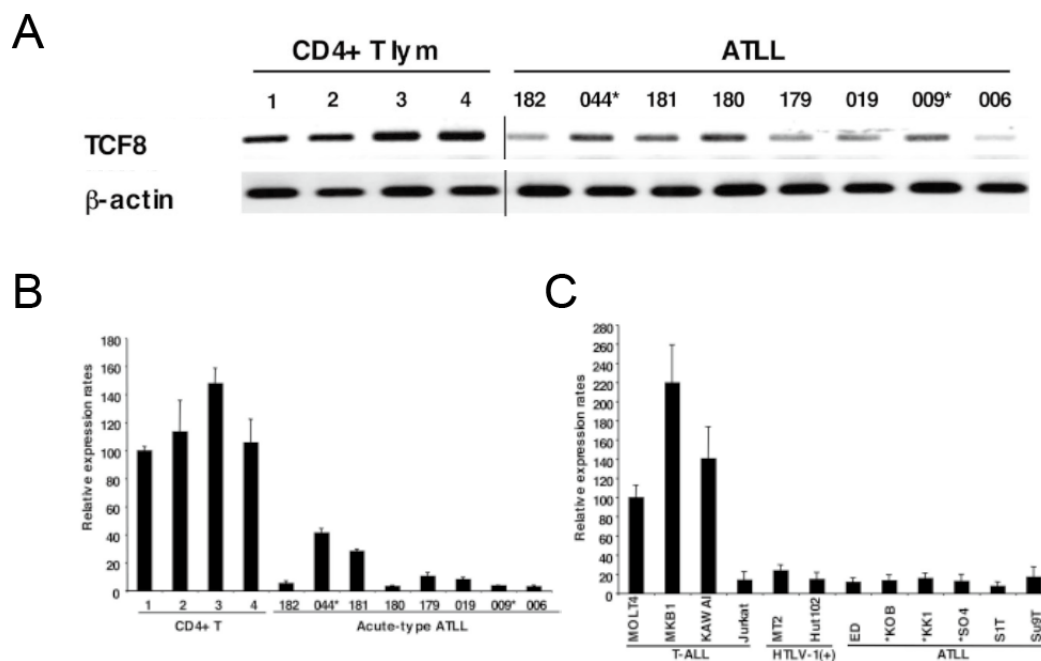


Figure 28. Down-regulated expression of Zeb1 (TCF8) in ATLL cells. (A) The expression profiles of Zeb1 mapped within the deletion region at 10p11. Semiquantitative reverse-transcription PCR (RT-PCR) was performed to measure the expression of Zeb1 mapped within the deletion region. (B) Quantitative RT-PCR analysis of ZEB1 mRNA in 4 samples of CD4⁺ T lymphocytes from healthy volunteers and 8 samples of ATLL cells from patients. (C) Quantitative RT-PCR analysis of ZEB1 mRNA in various types of T lymphoblastic leukemia cell lines. MOLT4, MKB1, KAWAI, and Jurkat are T-lymphoid leukemia cell lines; MT2 and HUT102 are HTLV-1–infected cell lines; and ED, KOB, KK1, SO4, S1T, and Su9T are ATLL cell lines. Three ATLL cell lines (indicated by *) showed the deletion of chromosome 10p11.2 with ZEB1. Adapted from Hidaka et al. (2008).

5.2.4 Zeb1 and E-proteins

The E-protein Heb (encoded by *Tcf12*) was among the top 3 genes best coregulated with Zeb1 (refer to Article 2 Figure S1B) (Heng et al., 2008). Heb is well known for its important roles throughout T cell development (Jones and Zhuang, 2011), especially at the DP stage (Emmanuel et al., 2018), which further supports a role for Zeb1 at the DP developmental stage. Many similarities were observed in the phenotypes of *Tcf12*-deficient (D’cruz et al., 2010) and *Cellophane* mutant mice, particularly the susceptibility of DP cells to cell death and the impaired development of iNKT cells.

Moreover, microarray data from the ImmGen consortium suggest that *Tcf12* and Zeb1 are strongly coregulated (Figure 29), and ATAC-seq data predict that they control chromatin accessibility during thymic T cell development together with Gata3, Tcf1,

Lef1, and Zfp740 (Figure S4) (Yoshida et al., 2019). Altogether, these data suggest a

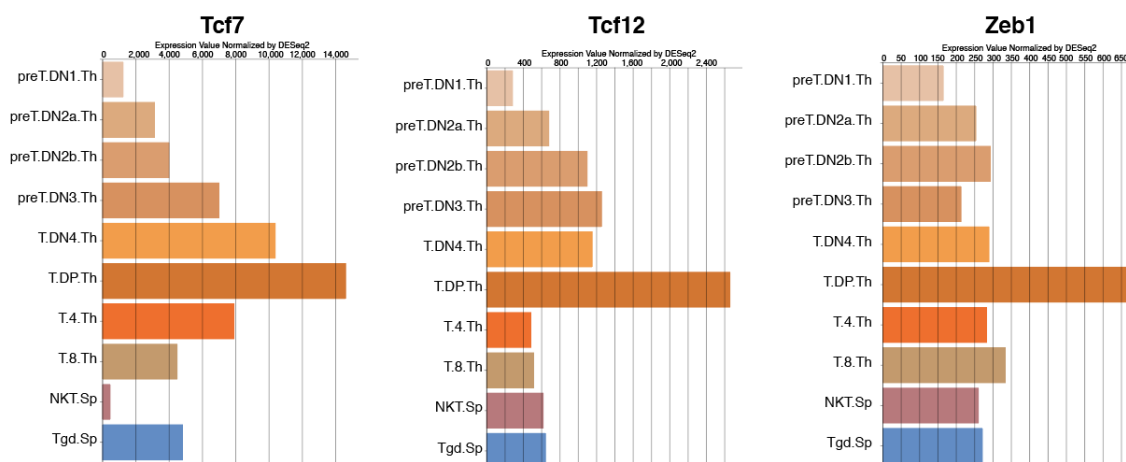


Figure 29. mRNA expression of Tcf7, Tcf17 and Zeb1. Microarray data extracted from Immgen consortium (<http://www.immgen.org>) on Tcf7, Tcf1 and Zeb1 expression during T cell development. Results show that the expression patterns of these three factors are similar, especially highest at DP stage.

strong functional link between Zeb1, Heb, and perhaps Tcf1 (also called Tcf7), which acts in coordination with Heb (Emmanuel et al., 2018). Similar to Tcf1 and Heb, Zeb1 is also known to bind tandem E-box motifs. A competition between Zeb1 and E proteins for the same binding sites has already been suggested to play a role in the control of GATA3 expression in human CD4 T cells (Grégoire and Roméo, 1999). Such a competition has also been established in the context of the CD4 enhancer, which is repressed by Zeb1, through competition with Heb for E-box binding (Brabletz et al., 1999). Moreover, Zfh-1 and Daughterless, the Drosophila homologs of mouse Zeb1 and Tcf12, are also known to compete for the same genomic sites (Postigo et al., 1999). These observations support a model whereby Zeb members and E proteins compete for DNA binding at dedicated motifs.

Moreover, by using a conditional knock-in mouse model expressing ET2 (a fusion protein between the transactivation domains of E-protein E47 and the bHLH domain of SCL/Tal1), it was demonstrated that E proteins could control a novel checkpoint that regulates the number of iNKT precursors resulting a block in the development of iNKT1 cells and a parallel increase in the iNKT2 and iNKT17 cells, which recapitulates the phenotype in *Cellophane* mutant mice (Hu et al., 2013). Further work will be needed to precisely map the interactions between Zeb1 and E proteins. The regulatory network may also include inhibitors of the differentiation genes Id2 and

Id3, which are TFs that bind and inactivate E proteins, thereby regulating their function. Id2 deficiency impairs hepatic iNKT cell survival (Monticelli et al., 2009). Moreover, a deficiency in Id3 has the same impact on NK1.1⁺γδ T cells as a deficiency in TCR signaling (Alonzo et al., 2010), which indicates a links between both factors.

5.2.5 Zeb1 and iNKT cells

iNKT cell development includes discrete stages (stages 0–3) that can be discriminated according to CD44 and NK1.1 expression (Benlagha et al., 2002; Pellicci et al., 2002). Three functionally distinct iNKT cell subsets have also been identified: iNKT1 cells, which express T-bet and mainly secrete IFN-γ; iNKT2 cells, which express Gata3 and Plzf and secrete IL-4 and IL-13; and iNKT17 cells, which express Rorγt and secrete IL-17 (Lee et al., 2013). iNKT1 cells more or less correspond to stage 3 iNKT cells (Figure 30) and in *Cellophane* mice there are less

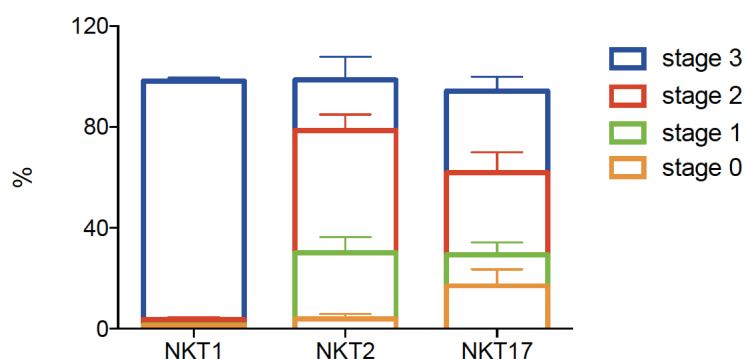


Figure 30. Constitution of different stages in iNKT subsets. iNKT cells were first divided into three populations based on different transcription factor expression (T-bet, Rorγ, Plzf, Gata3). In different subset, maturation marker CD44 and NK1.1 were utilized to gate different stage2 or iNKT cells. iNKT cells were dominately occupied by cells at stage 3, while others consist of cells from all the four stages.

mature iNKT cells at stage 3 and less iNKT1 cells. Several other iNKT subsets have been defined such as IL-10-producing iNKT10 cells (Sag et al., 2014), and IL-21-producing Bcl-6⁺ NKT-FH (follicular helper) cells (Chang et al., 2012). The TCR signal strength during selection governs the development of iNKT cell subsets, with stronger signals required for iNKT2 and iNKT17 development (Zhao et al., 2018).

Different thymic iNKT subsets are thought to play distinct roles in the immune response on the basis of their cytokines. The diverse cytokines expressed by iNKT cells profoundly influence many other cell types including NK cells, DCs, B cells and T cells ([Brennan et al., 2013](#)). According to our study, we came to the conclusion that the disturbed TCR signaling in T cell progenitor would contribute to the disproportional iNKT cell subsets in both *Cellophane* and *Zeb1*^{Δ13/+} mice. However, whether cytokine expression is also dysregulated in *Cellophane* iNKT cells and further interferes with other cell functions both in homeostatic and inflammatory conditions remains an open question.

6 Conclusion and perspectives

6.1 Conclusion

Project 1: T-bet and Eomes in NK cell development

In this research project, we wanted to understand the relationship between T-bet and Eomes during NK cell development and maturation using transcriptomic and epigenetic approaches. Even though Eomes and T-bet are co-expressed in NK cells, the ratio between both factors changes during maturation from a dominance of Eomes in immature cells towards a dominance of T-bet in mature ones. Eomes was required for the first transition from CD11b⁻ to DP, while T-bet was required for the second transition DP to CD27⁻. Deletion of either factor induced expression of the other one, which indicates compensation between T-bet and Eomes (Figure 31). Both

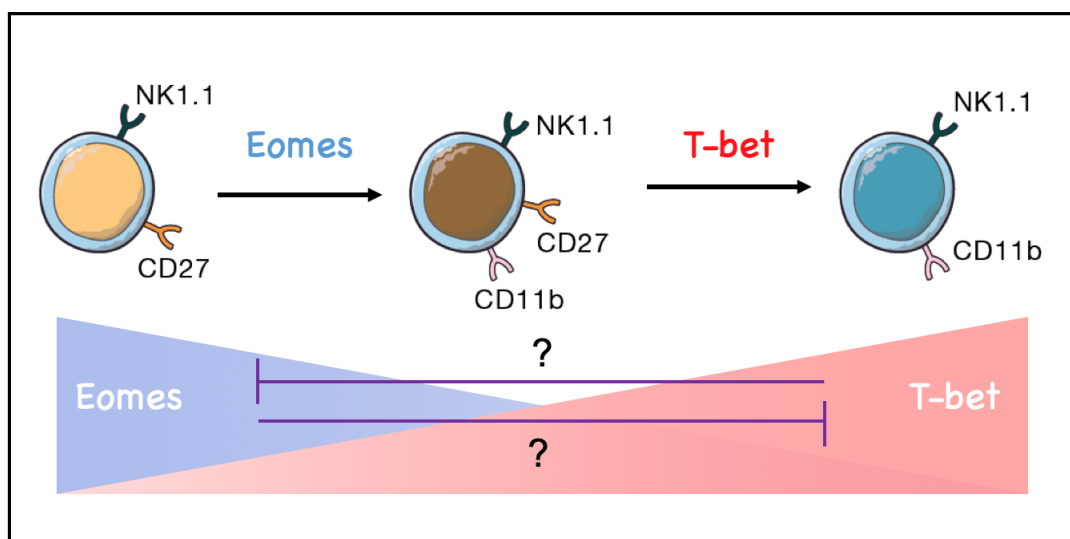


Figure 31. Eomes and T-bet are sequentially required for NK cell maturation. Eomes and T-bet controls different transitions during NK cell maturation. They also have complementary roles in the control of gene expression upon maturation as T-bet and Eomes regulate mostly distinct gene sets and at different maturation stages both transcriptionally and epigenetically. Whether T-bet and Eomes antagonize each other requires further investigation.

factors regulated cell proliferation and promoted survival sequentially. Eomes acts more often as an activator of transcription inducing the expression of many NK cell signature genes such as NK cell receptors at the immature stage, while T-bet acts more frequently as a repressor decreasing the expression of many T cell or

progenitor genes at the mature stage. The latter activity is associated with T-bet-specific ability to induce a series of transcriptional repressors such as Zeb2 and Blimp1. Using newly generated mouse models expressing endogenously-tagged T-bet and Eomes, we performed genome-wide analysis of T-bet and Eomes binding, and combined it with analysis of chromatin accessibility using ATAC-seq. This comprehensive analysis showed that Eomes and T-bet binding sites are largely overlapping, and motif analysis on peaks found by T-bet and Eomes suggested that both factors require binding to co-factors (e.g. Runx and Ets family) allowing TF-specific activity. Moreover, analysis of chromatin accessibility revealed that certain NK cell signature genes and maturation-acquired properties are largely dependent on Eomes and T-bet modulation. All in all, this study reveals a complementary role of Eomes and T-bet in NK cell maturation program.

Project 2: Zeb1 in iNKT cell development

In this project, we revealed the role and mechanism of Zeb1 in unconventional T cell development. In *Cellophane* mutant mice harboring a truncated form of Zeb1, we discovered a general decrease of unconventional T cells (i.e. iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49⁺ CD8 T cells) in terms of numbers and percentages in *Cellophane* mice while the number of conventional T cells was nearly normal at the periphery. These defects are associated with a major decrease in CD4⁻CD8⁻ DN stage 2 and

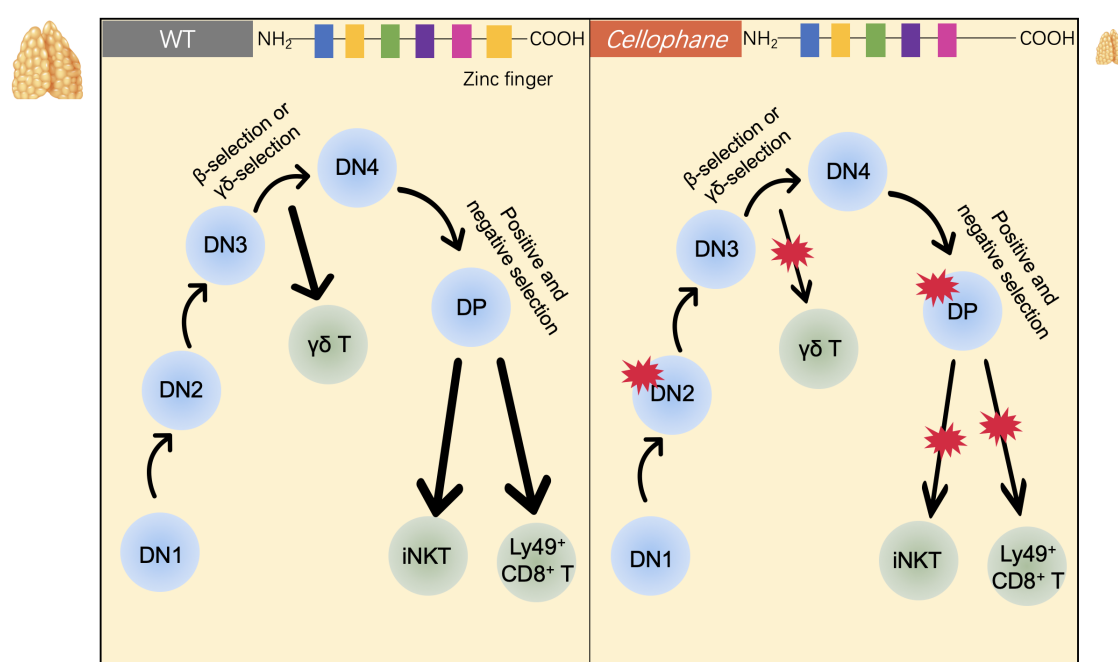


Figure 32. Zeb1 is essential for NK1.1⁺ T cell development. *Cellophane* homozygous mice virtually lack iNKT cells, NK1.1⁺ $\gamma\delta$ T cells and Ly49 expressing CD8 T cells. This specific defect in innate-like T cells is caused by a cell-intrinsic role of Zeb1 in T cell development. We show that Zeb1 regulates the transition to the SP stage by promoting cell proliferation, survival and repressing the expression of various molecules involved in modulating the strength of TCR signaling. Therefore, we propose that Zeb1 is a key regulator of thymocyte selection, essential for the development and survival of innate-like T cell subsets undergoing agonist-type selection.

CD4⁺CD8⁺ DP thymocytes as Zeb1 expression is induced in DN2 stage and is highest at DP stage among T cell subsets. Mechanistically, Zeb1 promoted survival and proliferation in both populations in a cell-intrinsic manner. Indeed, *Cellophane* DP displayed stronger signaling than WT DP upon TCR engagement in terms of calcium response, phosphorylation events and expression of early genes. A transcriptomic analysis of WT and *Cellophane* DP revealed that Zeb1 regulated the expression of

multiple genes involved in cell cycle and TCR signaling, possibly in cooperation with Tcf1 and Heb. Thus, Zeb1 is a key regulator of cell cycle and TCR signaling during thymic T cell development. We propose that thymocyte selection is perturbed in Zeb1-mutated mice, in a way that does not allow the survival of unconventional T cell subsets.

6.2 Perspectives

Project 1: T-bet and Eomes in NK cell development

1) Will the DNA binding profiles of T-bet and Eomes change upon NK cell maturation? By performing ChIP-seq analysis on bulk NK cells from T-bet-HAV5 and Eomes-HAV5 mice, we identified many interesting DNA loci that are associated with the two T-box factors. And many genes detected from ChIP-seq are transcriptionally deregulated as well according to the RNA-seq data. However, the data from bulk NK cells might create some bias as spleen NK cells are mostly mature NK cells. Comparing DNA binding sites in both mature and immature NK subsets would allow a better understanding of the relationship between T-bet and Eomes in NK cells.

2) Will there be any difference between T-bet and Eomes in terms of interacting partners? In our study, we demonstrated that T-bet and Eomes share lots of similarities and differences in terms of DNA-binding and transcriptional regulation. Therefore, we speculate that T-bet and Eomes might also have preferences in interacting with different co-factors (e.g. Runx3). And whether T-bet and Eomes can bind to each other would be an interesting point to clarify. With the advance of technology, the limited cell number would not be a problem anymore to address this point using mass spectrometry-based proteomic analysis.

3) Is there any difference in chromatin accessibility between the promoter and enhancer region in NK cells? Combining the ChIP-seq and ATAC-seq, we found that Eomes and T-bet separately regulate the epigenetic landscape of NK cells in different maturation stages. In-depth analysis is now required to study the promoter vs enhancer regions to get more information. Moreover, different histone hallmarks (e.g. H3K27ac3, H3K4me1) could be used to annotate the open chromatin loci as either activating or repressive to generate a more sophisticated map.

4) How does Eomes regulate NK cell circulation? We identified that Hobit, a master regulator of tissue residency, is negatively regulated by Eomes at the mRNA level. In conditional Eomes-knockout mice model, NK cell distribution in different tissues is dramatically disturbed. An in-vivo tracing system using anti-CD45 would probably be

ideal to determine whether the circulation of NK cells is perturbed in Eomes KO mice. Whether Hobit act downstream of Eomes to regulate NK cell circulation remains also an open question.

5) How does T-bet regulate NK cell effector functions? A functional annotation of T-bet regulated genes highlight cytokine responsiveness (e.g. IL-12). And it was confirmed that in the absence of T-bet, NK cells had the a defective ability to respond to IL-12 in terms of pSTAT4 induction and IFN- γ expression. RNA-seq data showed that IL-12R expression is dependent on T-bet, but we failed to detect the same effect at the protein level by flow cytometry (data now shown). Would T-bet deficiency dampen the responsiveness of NK cells to other cytokines (e.g. IL-21 and other)? A more comprehensive *in vivo* and *in vitro* analysis is needed to address this point.

6) How do T-bet and Eomes localize in NK cells? With our preliminary microscopic images, we realized that T-bet and Eomes largely colocalized in NK cells which raised questions about whether T-bet and Eomes localization determines the fate of NK cell development and maturation. It would be of great interest to track the T-bet and Eomes localization (i.e. cytoplasmic, nuclear, subnuclear) in NK cell subsets as T-bet and Eomes regulate different gene patterns. More information would be gained if histone markers were added in our analysis to discriminate the activating and repressive sites between NK subsets. Moreover, 3D images would definitely help elucidating the colocalization between T-bet and Eomes. A live imaging could also be interesting.

7) What is the respective roles of T-bet and Eomes in NK cell effector functions and polarization in disease models (e.g. MCMV)? So far, our study only focused on the role of T-bet and Eomes in NK cell in the naive situation. We are curious about how these two factors would work in disease models like virus infection and tumors. Would they display similar mechanisms of action in terms of DNA-binding, chromatin regulation and interaction with co-factors? All these points deserves further investigation.

Project 2: Zeb1 in iNKT cell development

1) Is there a role for Zeb1 in early T cell precursors? Zeb1 is an essential factor involved in EMT which is related to stemness and embryonic development. In addition, the fact that *Zeb1*^{-/-} mice die after birth raised the question of the relationship between Zeb1 and early T cell progenitor like common lymphoid progenitors (CLPs), and the possibility that the defect of T cell development in Zeb1 mutant mice might also involve early defects in BM progenitors.

2) Does Myb regulate Zeb1 expression and function? As discussed above, Zeb1 deletion in *Zeb1*^{Δ13/+} mice is reported to encompass a Myb binding site. To further document this, we could design specific oligos to perform oligo pull-down or EMSA assay to test the binding between Myb and the first intron of Zeb1 gene. Whether Zeb1 expression is disturbed in Myb knockout mice would be another point to study carefully at both mRNA and protein levels.

3) Are *Cellophane* and *Zeb1*^{Δ13/+} mutation loss-of-function and gain-of-function respectively? Zeb1 expression is increased in *Zeb1*^{Δ13/+} mice, while it is decreased in *Cellophane* mice. Our study found that *Cellophane* and *Zeb1*^{Δ13/+} mice have opposite phenotypes in terms of TCR signaling in DP, iNKT cell percentages in thymus. To obtain *Cellophane*^{+/-} X *Zeb1*^{Δ13/+} mice might be a way to better understand the two different mutations. Would the expression of Zeb1 and defect in T cells development be restored to normal?

4) Does Zeb1 regulate the T cell development at the epigenetic level? To understand if Zeb1 could directly regulate gene transcription, we retrieved the data from Immgen of all the OCRs that are predicted to have a binding site for Zeb1. By comparing with our transcriptomic data on DP cells, a strong overlap was found between the two lists indicating that Zeb1 could regulate gene expression through direct binding. However, a confirmation is needed using Zeb1 ChIP-seq or ATAC-seq of Zeb1 mutant cells to better map which genes are directly regulated, positively or negatively by Zeb1.

5) Are ILCs associated with splenic fibrosis in *Cellophane* mice? Several studies

have demonstrated that ILC cells participate in tissue fibrosis, and we found that ILC2 and ILC3 were increased in *Cellophane* spleen. But it remains to be clarified whether ILCs are the direct cause of the fibrosis and how ILCs affect fibrosis. Are cytokines secreted by epithelial cells or other immune cells like myeloid cells involved in fibrosis? Moreover, the decrease of ILCs in spleen was not observed in chimera mice. There might be two explanations for this. First, the survival rates of WT and *Cellophane* BM cells are uneven, which biases the results. Thus, the chimera assay with single donor from either WT or *Cellophane* BM is needed. Secondly, the dysregulation of ILCs are partly due to cell-extrinsic effect suggesting that epithelial cells can also contribute to ILC increase in *Cellophane* spleen.

6) Zeb1 and lymphoma? Deletion of Zeb1 is frequently found in lymphoma including CTCL and ATLL. In addition, we did observe few old *Cellophane* mice with enlargement of abdomen due to ascites, which could be due to lymphoma. It would be interesting to collect these ascites and measure the percentages of CD4⁺ T cells or other cell types. Additionally, we could also set up a lymphoma animal model using WT and *Cellophane* to test if Zeb1 mutated environment is more permissive for lymphoma progression.

7) Is Zeb1 important for iNKT cell function? In both *Cellophane* and *Zeb1*^{Δ13/+} mice, iNKT subsets were affected suggesting that the cytokines they express might be regulated by Zeb1. Different iNKT subsets have the capacity to produce different cytokines and further mediate immune functions of other immune cells. Therefore, it would be interesting to analyze the production of different cytokines by iNKT cells in the different mutant mice in different conditions.

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Annex



Review

T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1

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REVIEW**T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1**

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T-bet and Eomes are T-box transcription factors that drive the differentiation and function of cytotoxic lymphocytes such as NK cells. Their DNA-binding domains are highly similar, suggesting redundant transcriptional activity. However, while these transcription factors have different patterns of expression, the phenotype of loss-of-function mouse models suggests that they play distinct roles in the development of NK cells and other innate lymphoid cells (ILCs). Recent technological advances using reporter mice and conditional knockouts were fundamental in defining the regulation and function of these factors at steady state and during pathological conditions such as various types of cancer or infection. Here, we review these recent developments, focusing on NK cells as prototypical cytotoxic lymphocytes and their development, and also discuss parallels between NK cells and T cells. We also examine the role of T-bet and Eomes in human NK cells and ILC1s. Considering divergent findings on mouse and human ILC1s, we propose that NK cells are defined by coexpression of T-bet and Eomes, while ILC1s express only one of these factors, either T-bet or Eomes, depending on the tissue or the species.

Keywords: Eomes · ILCs · NK cell differentiation · T-bet · T-box transcription factors

Introduction

The T-box family of transcription factor (TFs) has been named after the discovery and cloning of the founding member Brachyury (short-tail in Greek) in a mouse line showing an altered tail development [1]. T-box proteins harbor a similar sequence-specific DNA-binding motif, or T-box domain, which spans 180–200 amino acid residues. The T-box family of TFs is subdivided in five fam-

ilies classified on the basis of overall homology. Most T-box TFs have important roles during embryogenesis and development and are evolutionarily conserved. T-box proteins function as transcriptional repressors or activators and some T-box TFs have both activation and repression domains that can function in different cellular or promoter contexts [2].

Loss-of-function mutations in the genes encoding for T-box TFs have been identified in many human syndromes associated with developmental defects [2]. T-bet and Eomesodermin (Eomes), encoded by *Tbx21* and *Eomes*, respectively, are the only T-box proteins expressed in the immune system. Their T-box DNA-binding domains present a strong degree of identity (Fig. 1A),

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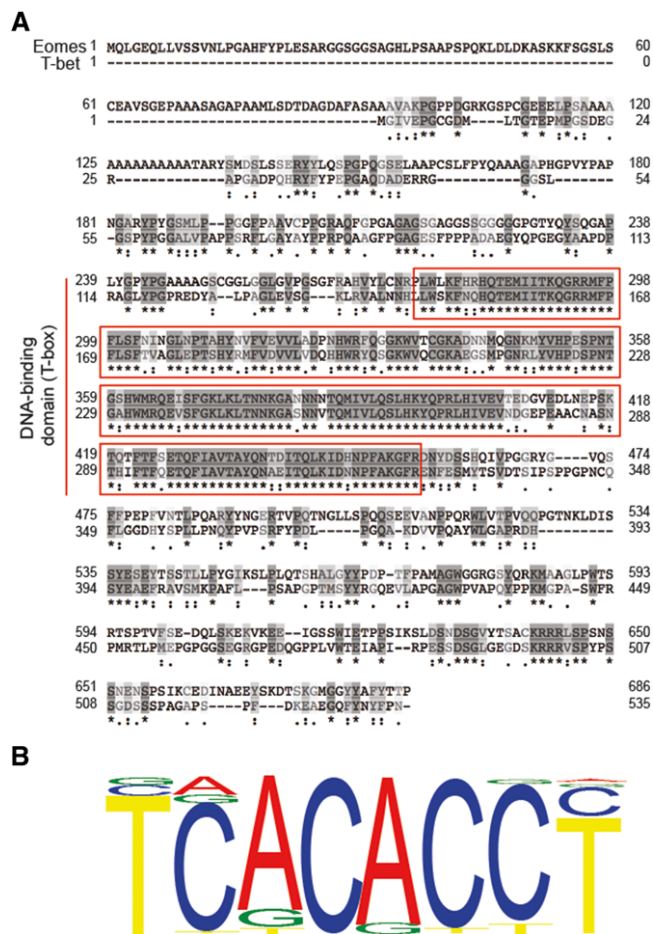


Figure 1. T-box domains of Tbet and Eomes and consensus DNA-binding site. (A) Alignment of human EOMES and T-BET sequences. The alignment was made using the CLUSTALO program on the Uniprot website. The red box corresponds to the T-box DNA-binding domain. Light gray shows amino-acid similarity and dark gray shows amino-acid identity. The overall identity between EOMES and TBET is 32%. Both T-box domains show 74% identity while the rest of the proteins is very different with about 16% identity. (B) Consensus DNA-binding site of *Xenopus* Eomes, as determined by sequencing oligonucleotides binding to in vitro translated proteins [135]. Letter size refers to Eomes-binding preference.

which suggests that they bind to the same DNA motifs (see Fig. 1B that shows for example Eomes consensus motif). By contrast, the sequence identity of other portions is quite divergent, suggesting that their interacting partners are distinct. Putative partners of T-bet and Eomes identified by high-throughput techniques support this hypothesis, as indicated in the reference database Biogrid [3].

T-bet and Eomes: Discovery and their role in mouse T-cell development

T-bet and Eomes have been first studied in T cells, in which their expression is inducible upon activation and differentiation. T-bet is the Th1-lineage-specifying transcription factor. It plays an important role in regulating IFN- γ production [4]. T-bet not

only promotes Th1 cell differentiation but also prevents the development of other T-helper cell lineages. Indeed, in the absence of T-bet, T-helper cells adopt the functional features of Th2 or Th17 cells because they fail to repress their genetic programs. T-bet expression is tightly controlled; IL-12 induces T-bet in a STAT4-dependent manner [5], while IFN- γ sustains T-bet expression, which results in a positive feedback loop during Th1 cell differentiation (see review [6]).

Eomes has first been studied in CTLs, where it was suggested that Eomes cooperated with T-bet to induce the expression of CD122, the beta chain of the IL-15 receptor, and to repress Th17-type cytokine expression [7]. CD122 is expressed by all cytotoxic lymphocytes and is essential for their development and survival [8]. Eomes is expressed at lower levels in CD4 T cells than CD8 T cells, and its role in CD4 T-cell functions remains ill-defined. Eomes overexpression rescues IFN- γ production by T-bet deficient T cells and promotes IFN- γ production [9–11] and cytotoxicity [12] in CD4 T cells. Like T-bet, Eomes can also inhibit IL-5 production in memory Th2 cells [13] and repression of Eomes is necessary for Th17 cell induction [14]. Therefore, most of the described roles for Eomes in CD4 T cells appear to be redundant with T-bet. Although it is generally assumed that T-bet and Eomes play comparable roles in T cells and NK cells, previous studies have clearly proved context-dependent function of these TFs in epigenetic regulation [15]. The following sections discuss more specifically the role of T-bet and Eomes in NK cells and Innate Lymphoid Cells (ILCs) development and function.

Murine NK cell development and maturation

NK cells are innate lymphocytes that contribute to the early defense against intracellular pathogens and to the immunosurveillance of tumors. They have been recently reclassified as members of group 1 ILCs and presented as the innate counterpart of cytotoxic CD8 T cells [16]. Indeed, they are endowed with perforin-dependent cytotoxic properties that can be enhanced upon activation by IL-15 [17]. NK cells also produce large amounts of IFN- γ rapidly after pathogen infection, as well as other cytokines and chemokines that have important roles during the early steps of the immune response [18]. This property is shared with other innate lymphocytes such as NKT cells, ILC1, and adaptive lymphocytes such as memory CD8 T cells that behave like innate lymphocytes during the first phases of infections [19]. NK cell activation is controlled by inhibitory and activating NK cell receptors expressed on the cell surface. Most inhibitory receptors bind to MHC class I molecules. Hence, MHC-I deficient cells are recognized and killed by NK cells. Three families of NK cell receptors recognize MHC class I: the primate KIR, the murine Ly49 receptors, and the CD94-NKG2 receptors shared by both rodents and primates. All three families encode both inhibitory and activating family members [20].

NK cells develop in the BM from NK cell precursors deriving from common innate lymphoid precursors [21]. Acquisition of the NK1.1 epitope marks their commitment to the NK cell lineage.

Next, they undergo a sequential maturation program that includes three discrete steps marked by surface levels of CD27 and CD11b. Immature CD11b⁻ CD27⁺ NK cells express high levels of NKG2A and low levels of Ly49 receptors. They are mostly represented in BM and LN. Upon acquisition of CD11b, NK cells massively proliferate in the BM [22]. CD11b⁺ CD27⁺ and CD11b⁺ CD27⁻ NK cells correspond to mature NK cells mainly found in the periphery, displaying the full repertoire of Ly49 receptors, and having the highest cytotoxic potential [23, 24]. At the CD11b⁺ CD27⁺ stage, NK cells acquire high expression of S1P5, which binds sphingosine-1 phosphate (S1P) that is carried by albumin and different blood and lymph lipoproteins, and induces their exit from the BM to the periphery [25]. In parallel, they also acquire the expression of CX3CR1 [26] whose ligand, fractalkine, is expressed on endothelial cells, and progressively lose the expression of CXCR3 and CXCR4 [27]; while the former is important for the entry into inflamed LN, the latter retains NK cells in the BM. This coordinated switch in chemotactic receptors impacts homing, and as a consequence mature NK cells are mostly circulating in blood, while immature NK cells are preferentially found within LN, BM, or nonlymphoid tissues such as the liver. Terminal maturation is also marked by the acquisition of other receptors such as KLRG1 [28] and CD146 [29], whose function in NK cells is not well defined.

Regulation and role of T-bet and Eomes during mouse NK cell development

Early NK cell development

Common ILC precursors lack T-bet and Eomes but express Id2, as shown using Id2 reporter mice [30]. Upon commitment to the NK cell lineage, the beta chain of the IL-2/15 receptor (CD122) is induced. This event occurs before the induction of NK cell receptors, and defines the NK cell precursor (NKp) [31] stage. Runx3 regulates Eomes expression in CD8 T cells and is also expressed in NKp stage as demonstrated using a Runx3-reporter mouse model [32]. Other factors such as NFIL3 [33, 34] Ets-1 [35], and TOX [36] may favor induction of T-bet and Eomes expression as both TFs are expressed when cells progress to the immature CD11b⁻ CD27⁺ stage. However, T-bet expression remains low until NK cells are released from the BM to the periphery. Interestingly, T-bet expression is actively repressed in the BM, and mature NK cells recirculating to this compartment rapidly decrease their T-bet expression [37]. This repression may be important to promote NK cell turnover as T-bet deficient NK cells display a high proliferation rate [38].

Regulation of CD122 and NK cell proliferation

At the NKp stage, T-bet and Eomes are weakly expressed in the BM, suggesting that CD122 induction is not firmly dependent on

T-bet and Eomes during commitment to the NK cell lineage, as it is the case of CD8 T cells [7]. Consistently, CD122^{hi} NKp and a few NK cells are present in mice deficient for both Eomes and T-bet [45]. However, T-bet and Eomes are essential for correct regulation of CD122 in mature NK cells in which CD122 expression is higher in T-bet deficient NK cells, along with Eomes [39]. Since CD122 expression is a rate-limiting factor for the use of IL-15, it seems that Eomes might be particularly important to promote NK cell proliferation via the induction of CD122, which would explain why there are so few peripheral NK cells in the absence of Eomes. These data may also suggest that T-bet represses CD122 expression in NK cells. However, CD122 is also expressed on NKT cells or ILC1s that express T-bet but not Eomes. To reconcile these seemingly contradictory observations, we propose a refined model for the control of CD122 expression in which both Eomes and T-bet would be capable to induce CD122, the former being more potent than the latter to transactivate CD122 expression, however, in this model, both TFs would be competing for the same binding sites. As T-bet represses Eomes expression, this model might explain why T-bet deficiency leads to high expression of both Eomes and CD122 [37] and high NK cell proliferation [38] (Fig. 2A).

Eomes promotes NK cell development and maturation

The role of Eomes in NK cell development has been recently addressed, when Eomes floxed mice became available. In these Vav1-Cre x Eomes^{fl/fl} mice, NK cell development is severely impaired, as evaluated by the absence of NK1.1 or NKp46 positive cells [40]. In the absence of Eomes, residual NK cells were originally considered as immature, but probably include many ILC1s. Since the conventional NK cell lineage is classically defined by Eomes expression, the discrimination between conventional but immature NK cells and ILC1s remains problematic in Eomes deficient mice. Despite this fact, most NK1.1⁺ cells in Eomes deficient mice express low amounts of CD11b and other maturation markers, suggesting that Eomes is required not only for NK cell development but also for NK cell maturation. Besides, transgenic expression of Eomes in ILC1s is sufficient to induce some attributes of conventional NK cells such as CD49b expression [41]. In the absence of Eomes expression (ILC1s or Eomes deficient NK cells), Ly49 receptors are weakly expressed, implying an important role of Eomes in either inducing the expression of these receptors or in opening the chromatin conformation at the Ly49 locus (leukocyte receptor complex). During MCMV infection, Eomes deficient Ly49H⁺ NK cells can expand to some extent, suggesting that inflammatory conditions may partially compensate for the lack of Eomes in NK cell expansion [40]. This proliferation may be promoted by IL-12 [42] as this cytokine is a potent inducer of T-bet. In turn, T-bet may induce CD25 (IL-2R α) expression on NK cells [43]. CD25 expression allows NK cells to use CD4 T cell-derived IL-2 [44] to sustain their proliferation upon infection, a process limited by regulatory T cells [45], which express high levels of CD25.

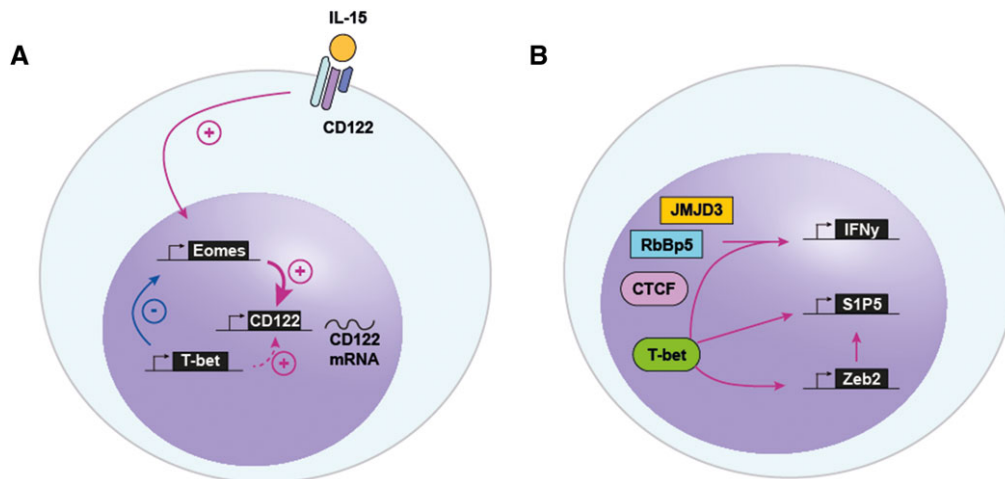


Figure 2. T-bet and Eomes regulatory mechanisms. (A) Regulation of CD122/IL2R β by T-bet and Eomes. In this model, both T-BET and EOMES would be able to bind to *Il2rb* promoter and promote transcription, but EOMES would be more potent than T-BET. As both TFs would compete for the same site, the relative amount of each TF would determine the level of CD122 expression. Moreover, CD122 expression would positively feedback on its own expression by inducing Eomes expression. (B) Regulation of IFN γ and S1P5 expression by T-bet in NK cells and CD8⁺ T cells. IFN γ and S1P5 are both regulated by T-bet. In T cells, it has been shown that IFN γ expression is turned on by promoting chromatin remodeling involving JMJD3, RbBp5 and CTCF co-factors. Given similarities between CD8 T cells and NK cells at many levels, IFN γ regulation is assumed to be similar in NK cells. S1P5 expression is induced through a positive feedback loop involving Zeb2: T-bet induces Zeb2 and Zeb2 binding on S1P5 promoter enhances T-bet binding and favors transcription.

T-bet promotes terminal NK cell maturation by cooperating with Zeb2

T-bet is known to be required for terminal NK cell maturation; in the absence of T-bet, NK cells express lower levels of CD11b and the sialoglycoprotein CD43, and have higher expression of the stem cell factor receptor c-Kit [38]. They also fail to downregulate CD27 [46] and to upregulate KLRG1 expression [47]. As discussed above, T-bet is essential for inhibiting NK cell proliferation, likely through the control of CD122 expression. T-bet also promotes viability through unknown mechanisms. Moreover, T-bet controls NK cell trafficking; in the absence of T-bet, NK cells accumulate in the BM and within LN. This may be caused by a decreased expression of S1P5 [48], which is involved in NK cell exit from the BM and LN in both mouse [25] and human [49]. Increased expression of chemokine receptors CXCR3 and CCR2 in the absence of T-bet may also contribute to this phenomenon [39]. Reduced numbers of NK cells in the spleen and lung of T-bet deficient animals may account for the reduced regional control of B16 melanoma [50] or *Toxoplasma gondii* [51] upon challenge.

In an effort to identify other TFs involved in NK cell maturation, we recently showed that the Zinc finger containing protein Zeb2 was also essential for NK cell maturation [39]. Zeb1 and Zeb2 are TFs that can induce epithelial-to-mesenchymal transition, an important process for embryonic development and for tumor metastasis [52]. Zeb1 has been previously shown to be involved in NK cell development [53] whereas Zeb2 expression is strongly induced during NK cell maturation. The phenotype of T-bet and Zeb2-deficient NK cells is very similar, suggesting a cooperation between these factors in NK cell maturation. T-bet was found to induce Zeb2, and Zeb2 was important to sustain T-bet

transcriptional activity, in a classical feed-forward loop (Fig. 2B). Overexpression of Zeb2 in T-bet deficient mice partially restored NK cell maturation, suggesting that some of the roles attributed to T-bet may be indirectly mediated via Zeb2 [39]. Blimp1 may also be part of the T-bet network, as a previous study identified that this TF is downstream of T-bet [54]. In addition, T-bet deficiency leads to attenuated IL-15-dependent Blimp-1 expression, hence controlling the peripheral maturation and proliferation of NK cells [54]. Blimp1 [55] and Zeb2 [56, 57] are also essential players in the control of CD8 T-cell differentiation, which highlights the similarity in the transcriptional mechanisms controlling NK and CD8 T cells. In effector CD8 T cells, in the absence of Zeb2, T-bet binding is altered on many promoters, suggesting that T-bet and Zeb2 act together and co-regulate the expression of several target genes. In few cases, Zeb2 promotes T-bet binding that results in promoter transactivation, while in most cases Zeb2 prevents T-bet binding, thereby repressing the expression of the target gene [56, 57].

Control of T-bet to Eomes balance

In the periphery, Eomes expression progressively decreases during NK cell differentiation, while T-bet expression steadily increases. This is reminiscent of effector CD8 T-cell differentiation [58] and indicates that high T-bet to Eomes ratios may favor differentiation over proliferation. Whether T-bet and Eomes levels are controlled by extrinsic signals in NK cells at steady state is unknown. Many cytokines and other signals regulate their expression upon activation. For example, IL-12 is a well-described inducer of T-bet in NK cells [38], while Eomes expression is induced by strong IL-2 signaling in T cells [59]. IL-4 and type I IFN have also been shown

to induce Eomes expression in innate memory T cells [60, 61], even though they have limited effect on Eomes expression in NK cells (TW, unpublished data).

The immunosuppressive cytokine TGF- β inhibits NK cells bioenergetics metabolism and negatively regulates both T-bet and Eomes, with a more pronounced effect on T-bet [62]. This effect could be mimicked by rapamycin, a specific inhibitor of the mTORC1 complex, which highlights the role of mTOR complex in T-bet and Eomes regulation. In CTLs, IL-12 induces a strong mTOR activation, which is required to induce T-bet, while mTOR inhibition decreases T-bet and leads to high Eomes expression [63]. At late stages of MCMV infection, i.e. during the virus clearance phase, inhibition of mTOR or activation of AMPK increased autophagic activity in NK cells and enhanced memory NK cell numbers through an Atg3-dependent mechanism [64]. Similarly, deficiency in Rictor, a key component of the mTORC2 complex, leads to enhanced memory T cell formation upon infection and higher Eomes expression, suggesting that mTORC2 induces T-bet and represses Eomes [65]. mTORC2 may act via Foxo1 and Foxo3, which are known regulators of T-bet in T cells [66] and NK cells [67]. The precise interplay between cytokines, mTOR and T-box factors in NK cells remains however to be fully elucidated. Subtle variations in T-bet/Eomes ratios may also implicate microRNAs that have a strong impact on NK cell maturation [68].

Genome wide analyses of T-bet and Eomes binding

Our understanding of the role of T-bet and Eomes in NK cell transcription and development, is mainly based on the comparison of gene expression between control and T-bet deficient NK cells [39], and between Eomes negative ILC1s and conventional Eomes positive NK cells [37, 69], respectively. ChIP and sequencing (ChIPseq) experiments have been performed for T-bet in mouse [56, 70, 71] and human [72–74] T cells and for Eomes in human embryonic stem cells [75], however, only one study reported the results of a T-bet ChIPseq experiment in NK cells [76], with limited analysis of the results. The context-dependent binding characteristics of these TFs doesn't allow a certain assumption that T-bet and Eomes operate the same way in T and NK cells. Moreover, T-bet and Eomes likely influence each other's binding to DNA, and a clear picture of their respective transcriptional activities should consider the expression levels of each of these TFs during NK cell differentiation or activation. Genome wide analyses in different NK cell subsets will be important to identify the mechanisms of T-bet and Eomes activities in NK cells, and, in particular, if they compete for the same motifs during development, maturation, and activation.

T-bet and Eomes in mouse NK cell effector functions

Cytokine secretion

In T cells, IFN γ , the hallmark Th1 cytokine, is directly induced by T-bet as shown by ChIP followed by microarray or luciferase

reporter assay [4, 15]. Cooperating physically with both the histone H3K27-demethylase and the histone H3K4-methyltransferase [77], T-bet promotes IFN γ transcription through its interaction with RbBp5 and JMJD3 [78, 79]. T-bet facilitates CTCF binding and chromatin looping respectively at +1kb and +66kb loci [80]. While the former have been shown in T cells, the corresponding mechanisms are likely similar in NK cells, given the overall similarity between NK and T cells in terms of IFN γ regulation (Fig. 2B). Yet, in NK cells, IFN γ expression is surprisingly normal in the absence of T-bet [81], except for a somewhat reduced ability to produce this cytokine in response to IL-12 and IL-18 [38]. Reciprocally, Eomes deficient NK cells expressed IFN γ at near-normal levels upon stimulation with IL-12 and IL-18 [40]. Thus, although T-bet and Eomes might have important roles in driving IFN γ expression in specific contexts, the current data suggest that other TFs may be sufficient to promote IFN γ expression in NK cells in response to cytokines and to remodel the IFN γ locus during their differentiation. The recent identification of Eomes negative ILC1s (see below) has prompted their functional comparison with Eomes positive NK cells. The finding that, in addition to IFN γ , ILC1s can produce larger amounts of TNF, IL-2, and GM-CSF [37] than NK cells implied that Eomes may be important to further specify the NK cell lineage and restrict the range of cytokines they can produce.

Cytotoxicity

While T-bet is dispensable for NK cell cytotoxic activity in vivo [36], Eomes by contrast seems important to acquire full cytolytic machinery. Indeed, Eomes may regulate the expression of several NK cell receptors such as the Ly49 receptors in mouse [40] and also activates transcription of *Gzmb* and *Prf1* [7, 82, 83]. As previously shown for memory CD8 T cells, Eomes could facilitate histone acetylation at the *GZMB* and *PRF1* locus in NK cells [84]. Accordingly, Eomes negative ILC1s express lower levels of granzymes and perforin compared to conventional NK cells [37]. This observation has led to the proposal that NK cells constitute the cytotoxic arm of the ILC family, while all other subsets are rather “helper” cells [16]. Yet, mice in which Eomes is deleted in NK cells seem to normally eliminate adoptively transferred MHC-I deficient cells [40], suggesting that ILC1s may retain in vivo cytotoxic activity.

Migration/trafficking

As discussed above, S1P5 is the dominant S1P receptor in NK cells and promotes egress from LN and BM [25]. In CD4 and CD8 T cells, T-bet acts directly on S1P5 expression by binding a conserved element 3' of S1P5 gene [48] and also by binding an additional upstream region [56]. Whether T-bet behaves the same way in NK cells needs to be further illustrated. The promoter region of another chemokine receptor CXCR3, which is involved in NK cell entry to inflamed LNs [85], was recognized as a direct-binding site for T-bet in NK cells [15, 86].

T-bet and Eomes in the development of mouse ILC1s

The existence of a peculiar NK cell population in the mouse liver has been recognized for many years. These cells were originally defined on the basis of their high expression of Trail, their low expression of CD49b (the target of the DX5 antibody) and their abundance in newborn mice [87]. Although they were initially described as precursors of classical Trail⁻DX5⁺ NK cells [40, 87], they are now defined as a separate ILC1 lineage, dependent on T-bet but not Eomes, the latter not being expressed in mouse ILC1s [37, 69]. Hepatic ILC1s are tissue-resident cells and do not depend on NFIL3 for their development. Given the plasticity of ILC populations, hepatic ILC1s might differentiate into NK cells under inflammatory conditions, even though this would require a strong induction of Eomes, whose expression is inhibited by T-bet [37]. Multiple other ILC1s populations have been described in the gut, in the salivary gland, in the uterine tissue or in fat pads that resemble hepatic ILC1s, with some tissue-specific characteristics [88]. By analogy with hepatic ILC1s, these cells may differentiate in situ and reside within tissues. Tissue-resident ILC1s all express T-bet at high levels and are presumably dependent on this factor for their development. As discussed above, transgenic expression of Eomes in ILC1s converts them at least in part into conventional NK cells [41]. Reciprocally, transgenic expression of T-bet promotes the development of ILC1s at the expense of NK cells [37] in organs such as the BM, in which they normally do not develop. This highlights the importance of T-bet to Eomes ratio in the early commitment of progenitors to the ILC1 versus NK cell lineage. Tissue-specific factors may favor one TF over the other to promote either lineage development. While the global absence of ILCs may not lead to overt immune deficiencies [89], recent studies have highlighted a possible role of ILC1s in the defense against pathogens and in anti-tumor responses [90, 91].

T-bet and Eomes in human NK cells and ILC1s

Human ILC1s have been first identified in mucosal tissues as T-bet⁺ Eomes⁻ ILCs capable of producing Th1 cytokines but devoid of cytotoxic properties [92]. However, a series of studies described that the human liver contains a large ILC population that resembles in many aspects the mouse hepatic ILC1 population [93–96] but expresses high levels of Eomes and not T-bet. These cells also express high levels of CXCR6 and CD69, express lower levels of KIR receptors and appear to be less cytotoxic than conventional NK cells. Interestingly a recent study showed that NK cells with a similar CXCR6⁺Eomes^{hi}Tbet^{lo} phenotype were present in several human fetal organs including the BM, the lung and the spleen [97]. The authors further showed that Eomes^{hi} Tbet^{lo} NK cells are differentiated before Eomes^{lo} Tbet^{hi} NK cells during in vitro cultures of hematopoietic stem cells. Moreover, when analyzing NK cell maturation stages on the basis of CD34/CD117/CD94/CD16 expression as previously described [98], immature NK cells were found to have high Eomes expression, which was decreasing with a progression of matu-

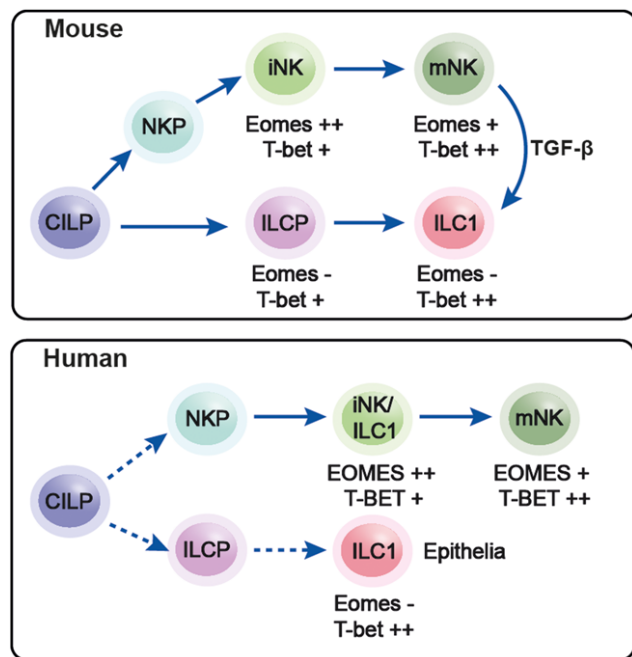


Figure 3. NK cells and ILC1 developmental pathways in mouse and human, with relation to T-bet and Eomes. In mouse, NK cells and ILC1 are independent lineages. NK cells are defined by Eomes expression, which is induced at the immature stage. Upon maturation, T-bet expression is enhanced and represses Eomes. Moreover, in tumor microenvironments or in the salivary glands, TGF- β may promote the conversion of NK cells into ILC1 by suppressing Eomes expression. In human, immature NK cells defined as Eomes^{hi} Tbet^{lo} express many of the attributes of “classical” epithelial Eomes⁻ Tbet^{hi} ILC1s. Both subsets may therefore be considered as ILC1s, from a functional point of view. In this model, mature NK cells would be defined by coexpression of high levels of both T-bet and Eomes, while ILC1s would express only one of them, either T-bet or Eomes. CILP: common innate lymphocytes precursor; ILCP: innate lymphoid cell precursor; NKP: NK cell precursor.

ration. Altogether, the authors proposed that CXCR6⁺ EOMES^{hi} TBET^{lo} NK cells correspond to immature NK cells, and that, like in the mouse, NK cell maturation is coupled with a decrease in Eomes and an increase in T-bet expression [97]. In this model, ILC1s would be precursors of mature NK cells, and this differentiation would be restricted by tissue-specific factors in organs like the liver. Such a differentiation would also change homing and trafficking properties, since NK cells are blood-circulating cells, while ILC1s are tissue resident. To reconcile divergent findings, we propose that coexpression of high levels of T-bet and Eomes defines mature NK cells, that are mainly found in the blood circulation and the spleen, while ILC1s are defined by single expression of T-bet or Eomes, depending on the tissue (Fig. 3). Further work will be required to precise the relationships between NK cells and ILC1s in all tissues.

The relationship between hepatic NK/ILC1s and memory/adaptive NK cells [99] that are known to home to the liver in mouse [100, 101], is another question that remains to be addressed. The precise phenotype of mouse adaptive NK cells remains unclear. In human, a recent study reported the identification of a population of CD49a⁺ EOMES⁻ TBET⁺ NK cells with

clonal-like properties in the liver that might correspond to liver-homing adaptive NK cells [102].

Regulation of T-bet and Eomes expression and their role during infection

T-bet and acute infections

Interest in the role of T-bet during infection started after it had been initially described that this TF regulated Th1 polarization and IFN- γ production by both T cells and NK cells [4]. In turn, IFN- γ upregulates T-bet in a positive feedback loop. One could argue that the concomitant skewing of Th1 commitment and dampened T-cell effector functions observed in *Tbx21*^{-/-} mice, contribute to the differential susceptibility to several pathogens that was observed in numerous studies. Indeed, T-bet is required for the control of *Mycobacterium tuberculosis* [103], *Salmonella typhimurium* [104], Vaccinia virus [105], and *Toxoplasma gondii* [51]. During the acute phase of infection with *Toxoplasma gondii*, T-bet is strongly induced in both T cells and mature NK cells [51]. Whereas MCMV infection also leads to T-bet induction in NK cells [106], *Tbx21*^{-/-} mice show no alteration of viral control. MCMV resolution being NK dependent, this implies that NK cells harbor sufficient residual anti-viral activity in the absence of T-bet. The increased susceptibility to other pathogens observed in *Tbx21*^{-/-} mice may not be only due to impaired cytotoxic lymphocyte responses. Indeed, T-bet was also shown to be induced in B cells and dendritic cells after IFN- γ stimulation [107] promoting respectively Ig class switch and Th1 priming.

Eomes and acute infections

Little knowledge regarding the role of Eomes was acquired in the context of infections, since Eomes floxed mice were only recently obtained [108] and because Eomes-deficient mice are not viable [109]. Reiner and colleagues showed that in the absence of Eomes expression in hematopoietic cells, Ly49H positive NK cells are still capable to expand to some extent upon infection with MCMV [38]. These Ly49H positive cells also upregulate CD11b and KLRG1 expression, albeit at lower levels than control NK cells. These data suggest that in the context of acute infections, other TFs may partially compensate for the loss of T-box factors in NK cells, likewise to T cells [58]. However, further studies are required to address this point in more details.

Chronic infections

NK cell effector functions have been shown to be impaired during several chronic infections, leading to the concept of NK cell “exhaustion,” as recently reviewed [110]. The regulation and role of T-box TFs have not been addressed in this context. By contrast,

an abundant literature is available on the transcriptional landscape associated with T-cell dysfunction during chronic infections (reviewed in [111–114]) and is discussed here considering the similarities between CD8 T cells and NK cells. T-bet is downregulated in virus-specific CD8 T cells [115] during chronic infection, resulting in severe exhaustion, a state of T-cell dysfunction. Using the mouse LCMV chronic infection model, it has been demonstrated that low expression of T-bet leads to a high expression of the inhibitory receptor PD-1, since T-bet directly suppresses PD-1 transcription. By contrast, the expression of Eomes is elevated in exhausted CD8 T cells [116]. Two subsets of exhausted CD8 T cells can thus be identified in chronically infected mice based on the expression of T-bet and Eomes: Tbet^{high} Eomes^{low} T cells that give rise to Tbet^{low} Eomes^{high} T cells. The Tbet^{high} subset is also characterized by an intermediate expression of PD-1 whereas the Eomes^{high} subgroup displays a higher expression of this immune checkpoint. In human, during HIV chronic infection, the balance between T-bet and Eomes also regulates the CD8 T-cell exhaustion process and the majority of HIV-specific CD8 T cells display a TBET^{dim} EOMES^{high} expression profile [117]. As shown in the LCMV model, high EOMES expression is associated with poorly functional T-cell phenotype. Interestingly, this elevated expression is not driven by stimulation with HIV antigens but rather by the level of inflammation [118, 119]. T-bet deficiency is also observed in virus-specific CD8 T cells from patients with chronic HBV or HCV infection [120] compared to those with acute infection. This indicates that T-bet is required and essential for a successful and appropriate T-cell response and to avoid the evolution into chronicity. T-bet has been shown to repress many type-I-IFN stimulated genes in T cells [71] and persistent production of type-I IFN has been implicated in promoting T-cell exhaustion, as recently reviewed [121]. It is therefore tempting to speculate that high TBET expression is important to counter the negative effects of type-I IFN on T-cell function. Further studies will be needed to test if this also applies to exhausted NK cells in the context of chronic infection. A recent paper described that exhausted NK cells in HBV individuals express lower levels of T-BET mRNA than NK cells from healthy donors [122], which is a first hint that similar exhaustion mechanisms may be at play in NK and T cells during chronic infections.

T-bet and Eomes regulation and role in anti-tumor responses

Tumor immune-surveillance is severely impaired in *Tbx21*^{-/-} mice, as firstly shown in the transgenic adenocarcinoma mouse prostate model [123]. Although the absence of T-bet did not impact the response against the primary tumor, T-bet was required to suppress metastasis development. This indicates that T-bet regulates the immune response against late steps of cancer development. These results were confirmed in a metastatic melanoma model [50]. Adoptive transfer of T-bet sufficient NK cells in *Tbx21*^{-/-} animals restored the anti-tumor response and conferred protection against melanoma. Moreover, as discussed

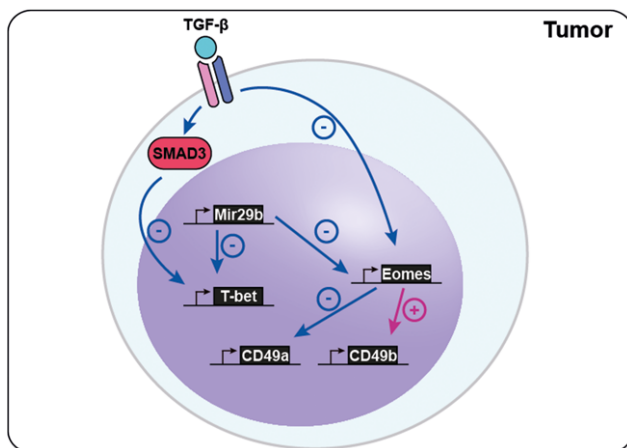


Figure 4. Regulation of T-bet and Eomes in the tumor microenvironment. Within the tumor microenvironment, several mechanisms may cooperate to suppress T-bet and Eomes expression by NK cells. Firstly, Mir29b is induced and targets the expression of both TFs. Secondly, TGF- β inhibits T-bet and Eomes expression via Smad3 binding. Eomes expression is important to repress CD49a and induce CD49b, which are often used as markers of ILC1s and NK cells, respectively.

above, T-bet and Zeb2 coregulate multiple target genes, and Zeb2 in NK cells has been shown to be essential for the control of metastatic melanoma [39]. Animals lacking T-bet and Eomes in T cells show several alterations in anti-tumor responses that indicate a critical role for these TFs in promoting CD8 T cells migration to the tumor tissue [124]. Upon adoptive transfer in tumor-bearing mice, NK cells progressively decrease the expression of both T-bet and Eomes. This correlates with a rapid loss of their effector functions. However, forced expression of Eomes, but not T-bet, in adoptively transferred NK cells led to a reduction in tumor burden and enhanced survival compared with mice treated with control NK cells [125]. The mechanisms used by tumor cells to inhibit T-bet and Eomes expressions are discussed below.

Role of TGF- β

It was suggested that TGF- β produced in the tumor microenvironment can diminish CD16 mediated IFN- γ production in a T-bet-dependent manner. Indeed, TGF- β can repress T-bet expression through phosphorylation of SMAD3 that binds T-bet promoter [126] (Fig. 4). Moreover, constitutive TGF- β signaling in transgenic mice blocks NK cell maturation at early stages, suggesting that both T-bet and Eomes are influenced by TGF- β signaling [62]. Furthermore, a very recent study also revealed that tumors may escape the NK cell immune-surveillance by a TGF- β dependent conversion of NK cells (CD49a⁻CD49b⁺Eomes⁺) into ILC1 (CD49a⁺CD49b⁺Eomes^{int}) that fail to control tumor progression [127]. SMAD4 could oppose this pathway in tumoral contexts as this TF was recently shown to restrain noncanonical TGF- β signaling mediated by the cytokine receptor TGF- β R1 in NK cells [128]. Zeb2, which is also essential for proper NK cell maturation might also contribute to this network. Indeed, Zeb2, also

known as SIP1 for Smad Interacting protein 1, contains a Smad-interacting domain that can potentially interact with all Smad members [129]. Under homeostatic conditions, TGF- β is dispensable for the differentiation of peripheral NK cell populations as revealed by NK-specific deletion of *Tgfb2* in mouse NK cells [62]. There is however one exception to this rule regarding salivary gland ILC1s, which express Trail, CD49a and others as a result of TGF- β mediated suppression of Eomes expression [130].

Other pathways

Micro-RNA29b, a negative regulator of both T-bet and Eomes (Fig. 4) was found to be upregulated in NK cells from leukemic mice and patients, which could contribute to this phenomenon [131]. The GSK3 kinase may also control T-bet expression in NK cells. Indeed, pharmacological inhibition of GSK3 induces an increase in T-bet, Zeb2, and Blimp1 expression in NK cells and greatly enhances their effector functions and anti-tumor potential [132]. A similar effect was observed in CD8 T cells upon combined blockade of CTLA-4, PD-1, and Lag-3 [133]. This highlights the important role of T-bet in coordinating different anti-tumor functions of cytotoxic lymphocytes and suggests that therapies aimed at restoring T-bet and/or Eomes expression in NK or T cells should prevent exhaustion of these cells.

Conclusions and outstanding questions

After more than 15 years of research on T-bet and Eomes, it has become clear that these related TFs have nonredundant activities despite their similarities, and that the balance between these two TFs determines the capacity of NK cells to proliferate, differentiate and affects a range of their effector functions. A wide array of cytokines and upstream TFs regulate their expression during NK cell development and in inflammatory conditions. In turn, T-bet and Eomes cooperate to promote NK cell maturation and homeostasis. Deregulation of either of these factors is associated with impaired immune responses during cancer or chronic infections. Despite recent advances, much remains to be learned on the spatio-temporal action of these TFs that are often coexpressed. Future studies will need to identify their respective target promoters and enhancers upon individual or combined expression. Proteomic analysis of interacting partners may also help to understand how they regulate transcription. In addition, since posttranscriptional regulation of these factors also occurs, it should be added to the picture in order to fully understand their mode of action. These modifications could affect the subcellular localization of T-bet and Eomes, which is known to be regulated upon T-cell activation [134]. New tools and reagents need to be generated to address these questions. Similar efforts should also be directed toward understanding other pairs of related TFs, which are also coexpressed in cytotoxic lymphocytes such as Foxo1 and Foxo3, Id2 and Id3, Runx1 and Runx3 or Zeb1 and Zeb2.

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Abbreviations: S1P: sphingosine-1 phosphate · TF: transcription factor

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