The Role of T-Box Transcription Factors in the Development and Plasticity of Natural Killer Cell Lineages

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Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
under the Executive Committee
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2016

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ABSTRACT

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Type 1 innate lymphocytes comprise two developmentally divergent lineages, type 1 helper innate lymphoid cells (hILC1s) and conventional NK (cNK) cells. All type 1 innate lymphocytes (ILCs) express the transcription factor T-bet, but cNK cells additionally express Eomesodermin (Eomes). We show that deletion of *Eomes* alleles at the onset of type 1 ILC maturation using NKp46-Cre imposes a substantial block in cNK cell development. Formation of the entire lymphoid and non-lymphoid type 1 ILC compartment appears to require the semi-redundant action of both T-bet and Eomes. To determine if Eomes is sufficient to redirect hILC1 development to a cNK cell fate, we generated transgenic mice that express Eomes when and where T-bet is expressed using *Tbx21* locus control to drive expression of Eomes codons. Ectopic Eomes expression induces cNK cell-like properties across the lymphoid and non-lymphoid type 1 ILC compartments. To investigate if T-bet is sufficient to direct type 1 ILC development into the hILC1 lineage, we also generated transgenic mice in which *Tbx21* locus control drives expression of T-bet codons. Enforced T-bet expression, however, does not appear sufficient to induce hILC1-like attributes among type 1 ILCs. Subsequent to their divergent lineage specification, hILC1s and cNK cells possess substantial developmental plasticity elicited by the absence or presence of Eomes.

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ACKNOWLEDGEMENTS

I would like to thank my thesis advisor and director of Columbia's MD-PhD program, Steven L. Reiner, for his mentorship. In particular, he has challenged me to become a better scientist and persevere in spite of obstacles. I am truly appreciative of the opportunity to improve my critical thinking skills and learn how to work independently during my tenure in the lab. I would like to thank all of my colleagues in the Reiner lab for their collaboration, especially Julie Chaix and Yen-Hua Chen for generating the transgenic mice characterized during my thesis work, as well as Amélie Collins for her valuable input over the years. I would also like to thank my colleagues in other labs on the ninth floor for their friendship and humor. I am grateful to the members of my thesis committee for their support and unwavering enthusiasm for my work: Ulf Klein, Kang Liu, Jennifer A. Punt, and Stephen G. Emerson. I would like to extend my gratitude to the MD-PhD program, especially Patrice Spitalnik, Ronald Liem, and Jeffrey Brandt for helping to make the transition to and from the graduate years as smooth as possible. I would also like to thank the Integrated Graduate Program in Cellular, Molecular, and Biomedical Sciences. My work would not have been possible without support from the National Institutes of Health Training Grant to Columbia's MD-PhD program, Grants R01 AI061699 and R01 AI113365 (to S.L.R.), and Grant F30 AI113963 (to O.P.). Finally, I would like to thank my friends and especially my family for always being proud and supportive of my endeavors and accomplishments. My friend, Lucas M. Bernard, for his advice and guidance over the years. My grandparents and other family members, who have provided me with love and numerous opportunities for growth by bringing me to America. My better half, Anatoliy, and his wonderful family for their unyielding love, support, and encouragement. I wouldn't be where I am today without you.

CHAPTER 1

INTRODUCTION

NK Cell Function

How do NK cells contribute to host immunity?

Natural Killer (NK) cells are white blood cells initially recognized for their intrinsic ability to mediate antitumor cytotoxicity (Herberman et al., 1975a; Herberman et al., 1975b; Kiessling et al., 1975a; Kiessling et al., 1975b). Like $CD8^+$ cytotoxic T cells, NK cells represent professional killer cells, but they can execute rapid killing without the need for specific immunization. NK cells are regarded as lymphocytes based on their morphology, origin, and expression of lymphoid antigens (Vivier et al., 2011). However, NK cells are considered to be part of the innate immune system because they lack antigen-specific recognition receptors and do not rearrange their receptor genes using recombination-activating gene (RAG) recombinases (Sun and Lanier, 2011). Unlike B cells and T cells, NK cells are present in normal numbers in mice deficient in RAG proteins (Mombaerts et al., 1992; Shinkai et al., 1992), although RAG protein expression has recently been implicated in NK cellular fitness and function at steady state and following viral infection (Karo et al., 2014). NK cells diversify their antigen-recognition repertoire by expressing germline-encoded receptors in a combinatorial fashion (Shifrin et al., 2014).

Since their initial discovery, NK cells have been found to mediate host responses to numerous pathogens, including viruses, bacteria, and parasites (Artis and Spits, 2015; Sun and Lanier, 2011). NK cells can be activated by contact-dependent signals from stressed somatic cells or by pro-inflammatory cytokines, such as IL-12 and type I interferons, that are secreted by other

immune cells (Sun and Lanier, 2011). NK cell activation triggers release of cytotoxic granules containing perforin and granzyme B, which allow entry of apoptosis-inducing effector proteins into target cells (Henkart, 1985; Russell and Ley, 2002). NK cells also express death receptor ligands, such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas-L, which allow them to initiate cytolysis upon engaging death receptors on target cells (Kayagaki et al., 1999; Zamai et al., 1998). Activated NK cells are major producers of IFN-γ, in addition to other cytokines (TNF-α and IL-10), growth factors (GM-CSF and G-CSF), and chemokines that facilitate recruitment of other immune cells to sites of inflammation (Vivier et al., 2011).

The role of NK cells in the immune response extends beyond their ability to kill harmful cells transformed by pathogens and tumors. NK cells selectively “edit” dendritic cell and macrophage populations to ensure that fully activated antigen-presenting cells display antigens to T cells and, subsequently, to attenuate the inflammatory response in a controlled fashion (Malhotra and Shanker, 2011; Moretta et al., 2005). NK cell-mediated cytotoxicity can also induce (Krebs et al., 2009; Robbins et al., 2007) or modulate (Andrews et al., 2010; Schuster et al., 2014) T cell and humoral responses. Although NK cells were originally identified for their “natural” killing abilities, optimal NK cell effector function appears to require priming by cytokines IL-12, IL-15, and IL-18 (Chaix et al., 2008; Guia et al., 2008; Lucas et al., 2007), as well as other contact-dependent signals from dendritic cells, macrophages, neutrophils, and T cells (Malhotra and Shanker, 2011; Mantovani et al., 2011). NK cell proliferation and activation is also dependent on IL-2, whose availability is promoted by $CD4^+$ T cells and restricted by regulatory T cells (Kerdiles et al., 2013). Therefore, the immune response necessitates bidirectional crosstalk between NK cells and other hematopoietic cells.

The notion that NK cells mediate rapid and short-lived responses independently of antigen-specificity has grown obsolete in recent years. Immunological memory of prior pathogen encounters, which results in enhanced responses upon pathogen rechallenge, has been regarded as a unique feature of the adaptive immune response mediated by B cells and T cells. There is now evidence to suggest that long-lived NK memory populations can mediate antigen-specific recall responses (Min-Oo et al., 2013). NK cells can mimic the $CD8^+$ T cell response to murine cytomegalovirus (MCMV). Subsequent to the activation, expansion, and contraction phases of the immune response, long-lived MCMV-specific memory cells are generated that can undergo secondary expansion, rapid degranulation, and IFN-γ production upon reactivation (Sun et al., 2009). Memory NK cells also appear to arise in response to cytokine stimulation with IL-12, IL-15, and IL-18. Restimulation of cytokine-induced NK memory cells results in robust IFN-γ secretion (Cooper et al., 2009). A subset of $CXCR6^+$ hepatic NK cells, furthermore, mediates memory in models of hapten-induced contact hypersensitivity and viral infection with antigens from HIV, influenza, and vesicular stomatitis virus (VSV) (O'Leary et al., 2006; Paust et al., 2010). NK cells, thus, appear to straddle the border between innate and adaptive immunity.

NK cells express receptors to distinguish healthy from diseased cells

NK cells express key receptors that regulate their activity by integrating inhibitory and activating signals transduced during interactions with target cells (Lanier, 2008; Orr and Lanier, 2010; Shifrin et al., 2014; Vivier et al., 2011). When NK cells encounter cells that express major histocompatibility class I (MHC-I) molecules, their effector function is inhibited (Karre et al., 1986). Healthy cells with abundant MHC-I expression are, therefore, more resistant to NK cell-mediated cytotoxicity than diseased cells with reduced MHC-I expression. Target cells whose

MHC-I expression is downregulated by viruses and tumors remain susceptible to attack by NK cells, but evade immunosurveillance by $CD8^+$ T cells (Lanier, 2008).

There are two families of murine NK cell receptors that bind to MHC-I antigens, which include Ly49 receptors and heterodimeric CD94-NKG2 receptors. NKG2 receptors are orthologous receptors in mice and primates, while primate killer immunoglobulin-like receptors (KIRs) are the functional homologs of murine Ly49 receptors (Orr and Lanier, 2010). Ly49C, Ly49I, Ly49G2, Ly49A, and CD94-NKG2A receptors signal through a common immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains (Lanier, 2008; Orr and Lanier, 2010). Ly49 receptors exhibit distinct specificities for polymorphic MHC-I molecules, while CD94-NKG2A receptors recognize the non-classical MHC-I molecule Qa1 (Orr and Lanier, 2010). Because NK cells express few Ly49 receptors during early life (Kubota et al., 1999), inhibitory interactions between CD94-NKG2A and Qa1 might contribute to the self-tolerance of fetal and neonatal NK cells (Sivakumar et al., 1999).

In contrast to inhibitory receptors, many activating receptors lack intracellular signaling motifs and associate with immunoreceptor tyrosine-based activating motif (ITAM)-containing adaptor proteins to propagate signals (Orr and Lanier, 2010; Vivier et al., 2011). Activating Ly49 receptors, such as Ly49D and Ly49H, are homologous to their inhibitory counterparts because they originated by gene duplication and conversion from inhibitory receptors (Orr and Lanier, 2010). Therefore, activating Ly49 receptors have the ability to bind to MHC-I or MHC-I decoy molecules (Abi-Rached and Parham, 2005; Orr and Lanier, 2010). Ly49H recognizes the MCMV-encoded m157 protein, an MHC-I decoy molecule, on the surface of cells infected with

the virus (Arase et al., 2002; Smith et al., 2002). Mice that lack Ly49H$^+$ NK cells to recognize MCMV-infected cells exhibited defective MCMV clearance (Daniels et al., 2001; Dokun et al., 2001; Lee et al., 2001).

NKG2D activating receptors recognize self-molecules that are scarce in healthy cells but become upregulated in unhealthy cells that are stressed, infected, or transformed by tumors (Lanier, 2005; Raulet, 2003; Raulet et al., 2013). NKG2D receptors are attractive targets for future cancer immunotherapies because NKG2D-mediated interactions are critical for the elimination of tumors by NK cells (Bauer et al., 1999; Cerwenka et al., 2001; Diefenbach et al., 2001). Furthermore, expression of Fc receptors allows NK cells to detect target cells coated with antibodies and triggers antibody-dependent cell-mediated cytotoxicity (Perussia et al., 1989). NK cells express numerous other activating receptors, such as the NK1.1 antigen and the natural cytotoxicity receptor NKp46, that have been implicated in host defense and loss of immune tolerance (Hudspeth et al., 2013). Their self-ligands in the host have yet to be clearly identified (Hudspeth et al., 2013; Shifrin et al., 2014), although NKp46 has been reported to interact with virus-derived hemagglutinins (Jarahian et al., 2011; Mandelboim et al., 2001).

Tuning responsiveness during NK cell education

The missing-self hypothesis provides a potential rationale for how NK cells recognize their targets. Shortly after the discovery of NK cells, it was suggested that MHC-I-deficient tumor cells were more susceptible to NK cell attack than their MHC-I-sufficient counterparts (Karre et al., 1986). Subsequent studies illustrated that NK cells could also attack non-tumor hematopoietic cells from syngeneic mice deficient in components of the MHC-I antigen

processing and presenting machinery, such as β2-microglubulin (β2m), MHC-I heavy chains, and TAP-1 (Bix et al., 1991; Dorfman et al., 1997; Liao et al., 1991; Ljunggren et al., 1994). Further support for the missing-self hypothesis came from studies that described NK cell-mediated rejection of MHC-disparate allogeneic bone marrow grafts (Murphy et al., 1987; Ohlen et al., 1990). MHC-I D^b bone marrow grafts that lacked expression of recipient MHC-I D^d molecules were rejected by NK cells in C57BL/6 (MHC-I D^b) mice that expressed the MHC-I D^d transgene (Ohlen et al., 1990). Introduction of the MHC-I D^d transgene in donor bone marrow protected the graft from rejection, suggesting that rejection occurred due to the absence of MHC-I D^d molecules in MHC-I D^b donor bone marrow. Conversely, MHC-I D^b mice that expressed the MHC-I D^d transgene acquired the ability to reject bone marrow grafts from MHC-I D^b donors (Ohlen et al., 1990). These findings extended the missing-self hypothesis in the context of the hybrid resistance phenomenon, which suggests that F1 offspring of inbred parental strains reject bone marrow grafts from either parent missing a full complement of maternal and paternal MHC-I components (Kumar et al., 1997). Hybrid resistance represented a major departure from the classical laws of solid organ transplantation because F1 hybrid progeny of MHC-I disparate mice accepted solid tissue, but not bone marrow grafts from either parent (Kumar et al., 1997). Rejection of solid organ grafts is largely mediated by recipient T cells (LaRosa et al., 2007). T cells in F1 hybrid progeny are tolerant of maternal or paternal MHC-I antigens and, therefore, do not reject parental solid organ grafts. In contrast, NK cells in F1 hybrid progeny recognize "missing self" on parental bone marrow grafts that lack expression of both maternal and paternal MHC-I molecules.

The missing-self hypothesis suggests that the absence of inhibitory self-MHC-I ligands is sufficient to break NK cell tolerance and render cells susceptible to NK cell attack. However, this notion appeared to be inconsistent with fundamental observations in the field. Based on the missing-self hypothesis, one might predict that NK cell hyperactivity and autoreactivity ensues in the absence of MHC-I expression. NK cells from MHC-I-deficient mice, however, were hyporesponsive and tolerant to MHC-I-deficient bone marrow grafts that were normally rejected by NK cells from MHC-I-sufficient mice (Bix et al., 1991; Hoglund et al., 1991; Liao et al., 1991; Vitale et al., 2002). A similar phenotype occurs when NK cells lack substantial expression of receptors that engage self-MHC-I ligands. There are three receptors specific for self-MHC-I (Ly49C, Ly49I, and CD94/NKG2A) in C57BL/6 mice, although interactions between Ly49A and the non-classical MHC-I molecule H2-M3 have also been reported (Andrews et al., 2012). Inhibitory receptors are expressed in a stochastic and probabilistic manner (Saleh et al., 2004; Tanamachi et al., 2001) and appear to be absent in a fraction of NK cells from C57BL/6 mice (Fernandez et al., 2005; Shifrin et al., 2014). In the absence of inhibitory receptors specific for self-MHC-I, NK cells exhibited hyporesponsiveness to tumors, MHC-I-deficient targets, and activating receptor stimuli (Andrews et al., 2012; Fernandez et al., 2005; Kim et al., 2005). Therefore, NK cell activation does not simply result from missing-self recognition and likely requires concurrent stimulatory input.

Three major models have emerged to explain NK cell education, which refers to the processes that determine the level of NK cell responsiveness and self-tolerance (Shifrin et al., 2014). The arming/licensing and disarming models are meant to justify the observation that NK cells with receptors specific for self-MHC-I are more responsive and cytotoxic toward MHC-I-deficient

targets, in contrast to NK cells that lack receptors specific for self-MHC-I. According to the arming/licensing model, a state of high responsiveness to MHC-I-deficient cells results from the ability of NK cells to interact with MHC-I^{+} cells (Shifrin et al., 2014). Prior to encountering MHC-I^{+} cells, NK cells are thought to remain immature, but this is not evident from their cell surface phenotype (Fernandez et al., 2005). In support of the arming/licensing model, it was suggested that functional competence can only be achieved by NK cells with inhibitory receptors that ligate host MHC-I molecules (Kim et al., 2005). Some studies demonstrated that constitutive or inducible expression of select MHC-I molecules enhanced the function of NK cells with cognate inhibitory receptors (Ebihara et al., 2013; Kim et al., 2005).

In contrast to the arming/licensing model, the disarming model suggests that NK cells have a baseline state of high responsiveness, but can be driven to enter a state of hyporesponsiveness or anergy by prolonged, unopposed stimulation (Shifrin et al., 2014). Support for the disarming model came from experiments in which mixed MHC-I-sufficient/ MHC-I-deficient bone marrow chimeras were challenged with MHC-I-sufficient and MHC-I-deficient bone marrow grafts (Wu and Raulet, 1997). Mixed bone chimeras generated using MHC-I-sufficient hosts exhibited an impaired ability to reject MHC-I-deficient bone marrow grafts. Mixed bone chimeras generated using MHC-I-deficient hosts were even more tolerant of MHC-I-deficient bone marrow grafts (Wu and Raulet, 1997). The presence of MHC-I-deficient hematopoietic and non-hematopoietic cells dominantly induced NK cell tolerance, which was consistent with the predictions of the disarming model. Another study utilized MHC-I transgenic mouse models to provide evidence in favor of the disarming model. Introduction of the MHC-I D^{d} transgene into all cells of C57BL/6 mice (MHC-I D^{b}) prompted NK cells to reject C57BL/6 bone marrow and lymphoblasts

(Johansson et al., 1997). NK cells from C57BL/6 mice with mosaic transgene expression, however, failed to respond C57BL/6 targets, suggesting that hyporesponsiveness was dominantly induced because the transgenic MHC-I ligand was not expressed by all cells (Johansson et al., 1997). Furthermore, other studies illustrated that continuous engagement of Ly49H and NKG2D receptors by their cognate ligands m157 and Rae-1, respectively, also induced NK cell tolerance in accordance with the disarming model (Champsaur et al., 2010; Oppenheim et al., 2005; Sun and Lanier, 2008b; Tripathy et al., 2008).

Unlike the arming/licensing and disarming models, the rheostat model suggests that NK cell responsiveness varies quantitatively and does not simply reflect responsive versus hyporesponsive states. The rheostat model proposes that responsiveness increases with the number of MHC-I-specific inhibitory receptors expressed by NK cells, as well as the affinity of inhibitory receptors for self-MHC-I ligands (Brodin et al., 2009; Joncker and Raulet, 2008). The frequency and strength of interactions between inhibitory receptors and their cognate MHC-I ligands might promote NK cell responsiveness by opposing prolonged stimulation or by aiding in the transduction of activating signals that have yet to be discovered (Orr and Lanier, 2010).

The tuning model for NK cell education is currently favored in the field because it integrates concepts from the arming/licensing, disarming, and rheostat models (Orr and Lanier, 2010; Shifrin et al., 2014). According to the tuning model, NK cell responsiveness is governed by the integration of stimulatory and inhibitory signals. Strong or weak net stimulation drives NK cells to their lowest or highest states of responsiveness, respectively, but NK cell responsiveness can subsequently be reset in response to changing steady state conditions. Wild-type NK cells

become hyporesponsive in MHC-I-deficient environments while MHC-I-deficient NK cells can regain functional competence in wild-type environments (Elliott et al., 2010; Joncker et al., 2010). NK cell responsiveness can, thus, be tuned even after maturation is complete, which cannot be explained by the arming/licensing model alone.

NK cells undergo a sophisticated, tunable education process that endows them with functional competence in response to specific ligands and prevents them from becoming autoreactive after persistent stimulation. The plasticity of NK cell responsiveness, however, could be harmful in the setting of infection when the protective role of NK cells hinges on their ability to remain reactive under inflammatory conditions (Shifrin et al., 2014). During infection with *Listeria monocytogenes*, unlicensed NK cells that lacked inhibitory receptors specific for self-MHC-I responded as strongly as licensed NK cells that expressed inhibitory receptors specific for self-MHC-I (Fernandez et al., 2005). Furthermore, unlicensed NK cells preferentially expanded and provided better protection than licensed NK cells during MCMV infection (Orr et al., 2010; Sun and Lanier, 2008a). An enhanced understanding of the processes that tune NK cell reactivity is critical for ensuring optimal use of NK cells during antiviral defense.

<u>*NK cells in human diseases*</u>

The importance of NK cells in the human immune response has become evident from studies of human NK cell deficiencies, some of which are rare isolated NK cell disorders while others comprise larger immunological syndromes (Orange, 2006; Orange, 2012). Human NK cell deficiencies result in extreme host susceptibility to infection with papillomaviruses and herpesviruses, such as herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein-Barr virus

(EBV), and varicella zoster virus (VSV) (Lee et al., 2007). Human NK cell deficiencies are categorized according to defects in development versus function, but identification of genetic lesions and mechanisms that result in specific NK cell defects remains challenging (Orange, 2012). Recent exome sequencing studies illustrated that a cohort of patients with NK cell deficiencies had mutations in the *GATA2* gene, which regulates development of human NK cells and other hematopoietic lineages (Dickinson et al., 2011; Jouanguy et al., 2013). GATA2 deficiency has been linked to severe viral infections, HPV- and EBV-associated neoplasms, and miscarriages subsequent to impaired control of viral infections, cancer, and homeostasis at the maternal-fetal boundary (Spinner et al., 2014).

Genetic analyses of MHC-I and KIR alleles in virus-infected individuals revealed that interactions between activating or inhibitory NK cell receptors and their cognate MHC-I ligands could have protective effects during infection. Activating KIR2DS1 receptors on some NK cell clones triggered NK cell cytotoxicity upon recognition of peptide-MHC-I complexes on the surface of EBV-infected cells (Stewart et al., 2005). Interactions between inhibitory KIR2DL3 receptors and cognate HLA-C1 ligands, furthermore, promoted resolution of hepatitis C virus (HCV) infection. The pairing between KIR2DL3 receptors and HLA-C1 ligands is thought to be protective because it results in transduction of weak inhibitory signals, allowing NK cells to remain in a state of high functional competence (Khakoo et al., 2004). NK cells that expressed stimulatory KIR3DS1 receptors, moreover, inhibited HIV-1 replication in target cells that expressed HLA-B Bw4 antigens, resulting in delayed progression to AIDS (Alter et al., 2007; Martin et al., 2002a). Genetic combinations of KIR3DS1 receptors with HLA-B Bw4 antigens have also been linked to autoimmune disorders, such as rheumatoid arthritis, psoriasis, type 1

diabetes mellitus, multiple sclerosis, and numerous others (Fogel et al., 2013; Fusco et al., 2010; Martin et al., 2002b; van der Slik et al., 2003; Yen et al., 2001). Deciphering the mechanisms that tune NK cell responsiveness will be critical for future therapeutic approaches to human infectious and inflammatory diseases.

NK cell-based immunotherapy in the treatment of malignant tumors is already underway (Cheng et al., 2013). The treatment of hematologic malignancies, such as leukemia, involves the use of allogeneic bone marrow stem cell transplantation. Donor cells from bone marrow allografts have the ability to kill remnant neoplastic cells after host chemotherapy, which contributes to improved survival rates following transplantation. This graft-versus-leukemia (GVL) effect is mediated by cytotoxic T cell alloreactivity against recipient minor and major histocompatibility antigens, which occurs when there are MHC mismatches between donors and recipients (Goldman et al., 1988; Horowitz et al., 1990). In addition to T cells, NK cells can also mediate GVL effects in the setting of MHC haplotype mismatch between donors and recipients. An optimal GVL effect is not likely to occur after transplantation of autologous NK cells whose cytotoxicity might be inhibited by interaction with self-MHC-I molecules (Farag et al., 2002; Ruggeri et al., 1999). It has yet to be determined whether inhibition of autologous NK cells can be overcome using antibodies that block interactions between inhibitory receptors and self-MHC-I ligands (Cheng et al., 2013). Based on the principle of missing-self recognition, allogeneic NK cells could mount a response against neoplastic recipient cells in the absence of self-MHC-I ligands to engage their inhibitory receptors. KIR mismatched allogeneic NK cells, thus, demonstrate superior antitumor cytotoxicity and protection against relapse. Current efforts

to improve NK cell-mediated antitumor immunotherapy are focused on expansion, activation, and genetic modification of NK cells in vitro for use in clinical practice (Cheng et al., 2013).

NK Cell Development and Homeostasis

Development of NK cell progenitors from early lymphoid progenitors

NK cells progress through multiple stages of maturation in the bone marrow before their functional competence can be tuned in the periphery. Like other blood lineages, NK cells originate from self-renewing hematopoietic stem cells (HSCs) (Yokoyama et al., 2004). HSCs differentiate into multipotent progenitors, which bifurcate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) after passage through a myeloid-lymphoid progenitor intermediate stage (De Obaldia and Bhandoola, 2015). NK cells originate from CLPs, which exhibit B cell, T cell, NK cell and dendritic cell differentiation potential in vitro and in vivo (Galy et al., 1995; Kondo et al., 1997). Beyond the CLP stage, lineage-specific mechanisms likely repress the myeloid potential of lymphoid progenitors (De Obaldia and Bhandoola, 2015). The Notch signaling pathway serves as one such example. A requirement for T cell development (Di Santo, 2006), Notch signaling also enhances NK cell development in some circumstances. Expression of Notch ligands Jagged1 and Jagged2 has been suggested to promote NK cell differentiation from lymphoid progenitors (DeHart et al., 2005; Jaleco et al., 2001; Lehar et al., 2005). Furthermore, Notch signaling facilitates the reprogramming of B cells and T cells into NK-like cells subsequent to deletion of the transcription factor Bcl11b (Carotta et al., 2006; Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b). Expression of B cell and T cell lineage-specifying factors might, therefore, be crucial for suppressing an alternative NK cell fate.

Early stages of NK cell specification and commitment have been elucidated in recent years. The earliest NK lineage-committed progenitors (NKPs) can be identified by expression of CD127 (IL-7Rα), CD27, and CD244. Absence of CD135 (Flt3) and CD117 (c-kit) expression further distinguishes NKPs from upstream CLPs (Carotta et al., 2011; Fathman et al., 2011). NKPs initially lack expression of CD122 (IL-2/IL-15Rβ), but upregulate CD122 at a later stage prior to the acquisition of NK lineage antigens NK1.1 and NKp46 (Rosmaraki et al., 2001). IL-15 signaling, therefore, plays a critical role in NK cell development after lineage specification (Kennedy et al., 2000; Vosshenrich et al., 2005). A minor subset of NKPs has also been identified in extramedullary organs (Di Santo, 2006; Ikawa et al., 2001; Rosmaraki et al., 2001), but it is not known whether these precursors arose in situ or circulated from the bone marrow. Bipotent T/NK cell progenitors have also been identified (Douagi et al., 2002; Ikawa et al., 1999; Michie et al., 2000), but they are thought to be dispensable for NK cell development (Di Santo, 2006).

Differentiation of NK cells from NK cell progenitors

Upon acquisition of CD122, NKPs differentiate into NK cells that are identifiable by expression of NK1.1 and NKp46, as well as continued expression of CD122. IL-15 is one of at least six cytokines, in addition to IL-2, IL-4, IL-7, IL-9, and IL-21, that functions by signaling through the common cytokine receptor γ-chain. NKPs develop normally in the absence of the common cytokine receptor γ-chain, but NK cell differentiation is severely impaired (Vosshenrich et al., 2005). Cytokines other than IL-15 appear to be dispensable for NK cell development because only genetic deletion of IL-15 phenocopies the NK cell defects in mice lacking the common cytokine receptor γ-chain (Vosshenrich et al., 2005). Moreover, IL-15 plays a critical role in NK

cell homeostasis and activation. NK cell survival and expansion are compromised when IL-15 fails to be expressed or presented *in trans* by accessory cells lacking IL-15Rα expression (Kennedy et al., 2000; Lodolce et al., 1998; Prlic et al., 2003; Ranson et al., 2003). The molecular basis for the importance of IL-15 in NK cell biology has recently been linked to regulation of the metabolic checkpoint kinase mTOR. IL-15 signaling activates mTOR, which stimulates NK cellular growth and nutrient uptake (Marcais et al., 2014). IL-15-mediated activation of mTOR sustains NK cell proliferation during bone marrow development, as well as inflammation and viral infection (Marcais et al., 2014).

Developing NK cells progress through multiple stages of maturation before their egress from the bone marrow into the periphery. As NKPs transition to immature NK cells, there is sequential acquisition of NKG2D, NK1.1, NKp46, and CD94-NKG2, as well as transient expression of CD27, TRAIL, integrin α_v (Chiossone et al., 2009; Kim et al., 2002; Takeda et al., 2005). As they progress to mature stages, immature NK cells upregulate expression of Ly49 receptors and integrin CD49b (DX5), but downregulate expression of TRAIL and integrin α_v (Chiossone et al., 2009; Gordon et al., 2012; Kim et al., 2002; Takeda et al., 2005). Mature DX5$^+$ NK cells undergo terminal stages of differentiation marked by induction of CD11b, CD43, and KLRG1, as well as repression of CD27 (Chiossone et al., 2009; Huntington et al., 2007; Kim et al., 2002). Terminal maturation appears to begin in the bone marrow, but is completed in the periphery (Chiossone et al., 2009; Kim et al., 2002).

Transcription Factors that Govern NK Cell Differentiation and Maturation

Factors that specify early stages of NK cell development

Mechanisms of lymphoid lineage specification from HSC descendants are incompletely understood. Early lymphoid priming likely depends on the activity of numerous transcription factors, such as HOXA9, STAT5, PU.1, E2A, Ikaros, Satb1, Ly1-1, in the bone marrow environment (De Obaldia and Bhandoola, 2015; Sun, 2015). Generation of CLPs, for instance, requires Ikaros-dependent suppression of self-renewal and multi-potency programs in differentiating HSCs (Georgopoulos et al., 1994; Georgopoulos et al., 1997; Ng et al., 2009; Papathanasiou et al., 2009). E proteins also support CLP development by attenuating myeloid potential and initiating RAG-dependent antigen receptor gene recombination (Borghesi et al., 2005; Dias et al., 2008; Igarashi et al., 2002; Karo et al., 2014; Schlissel et al., 1991; Yokota et al., 2003). Repression of E2A proteins by the transcription factor Id2 is necessary for the development of NK cells (Boos et al., 2007) and all other innate lymphoid cells (ILCs) (Eberl et al., 2004; Monticelli et al., 2011; Moro et al., 2010; Yokota et al., 1999). Prolonged E protein activity promotes preferential development of B cells at the expense of other lymphocyte lineages (Nutt and Kee, 2007).

NK cell lineage specification is regulated by multiple transcription factors, including ETS family members PU.1 and Ets-1, as well as NFIL3 and TCF-1. PU.1 indirectly contributes to NK cell development by inducing expression of CD127 and CD135 among hematopoietic progenitors (Carotta et al., 2010; DeKoter et al., 2002). Absence of PU.1, furthermore, resulted in a stronger reduction of NKPs than NK cells (Colucci et al., 2001). Remnant $PU.1^{-/-}$ NK cells did not exhibit substantial phenotypic changes, suggesting that PU.1 might be dispensable for later

stages of NK cell maturation (Colucci et al., 2001). In contrast, Ets-1 appears to be critical for NK cell lineage commitment, expression of numerous NK cell receptors, and attenuation of NK cell responsiveness (Barton et al., 1998; Ramirez et al., 2012). Unlike other factors, NFIL3 is a key regulator of NK cell development that does not strongly impact development of B and T lymphocyte lineages (Gascoyne et al., 2009; Kamizono et al., 2009). NFIL3 expression from the CLP to the NKP stages is necessary for the development of NKPs and NK cells (Firth et al., 2013; Male et al., 2014). NFIL3 drives NK cell development by directly inducing Eomes expression (Male et al., 2014; Seillet et al., 2014a). TCF-1, a regulator of T cell specification and differentiation (Germar et al., 2011; Weber et al., 2011), also contributes to NK cell lineage commitment, in addition to the early development of other ILC lineages (Yang et al., 2015). The transcription factors Id2 and TOX, however, appear to be dispensable for NK cell lineage specification despite their requirement for NK cell development (Aliahmad et al., 2010; Boos et al., 2007). It has recently been suggested that Id2 and TOX, in conjunction with TCF-1, control the emergence of multipotent lymphoid progenitors to all ILC lineages (Klose et al., 2014; Seehus et al., 2015; Yang et al., 2015).

Factors that promote NK cell differentiation and activation beyond the progenitor stages

Other transcription factors contribute to NK cell maturation subsequent to lineage specification. T-bet and Eomes are homologous T-box transcription factors that regulate the fate and function of $CD4^+$ and $CD8^+$ T cells (Banerjee et al., 2010; Intlekofer et al., 2008; Intlekofer et al., 2005; Pearce et al., 2003; Szabo et al., 2002). T-bet and Eomes, furthermore, appear to be genetically downstream of transcription factors responsible for the emergence of early innate lymphocyte progenitors (Male et al., 2014; Seehus et al., 2015; Seillet et al., 2014a; Vong et al., 2014; Xu et

al., 2015; Yu et al., 2014). Using mice with hematopoietic and conditional deficiencies of T-bet and Eomes, a previous study suggested that T-bet and Eomes regulate distinct checkpoints of NK cell development (Gordon et al., 2012). T-bet stabilizes immature-appearing Eomes^{-} cells marked by constitutive TRAIL expression (Gordon et al., 2012). Eomes is required for progression to a mature-appearing state characterized by repression of TRAIL, acquisition of CD49b, and induction of diverse Ly49 receptors (Gordon et al., 2012). Fetal NK cell hematopoiesis in the liver is restrictive to Eomes expression and, thus, progresses to the T-bet-dependent Eomes^{-} state (Daussy et al., 2014; Gordon et al., 2012; Takeda et al., 2005). In contrast, adult NK cell development in the bone marrow is restrictive to T-bet expression, which facilitates maturation of Eomes^{+} cells (Daussy et al., 2014; Gordon et al., 2012).

It has also been suggested that T-bet regulates NK cell homeostasis and trafficking to the periphery (Jenne et al., 2009; Townsend et al., 2004). Furthermore, T-bet contributes to terminal stages of NK cell maturation by promoting repression of CD27 and expression of KLRG1, CD11b, and CD43 (Gordon et al., 2012; Townsend et al., 2004). Genetic deletion of other transcription factors, such as GATA-3, IRF-2, Aiolos, Blimp-1, and Zeb2, phenocopies halted terminal maturation in T-bet-deficient mice (Holmes et al., 2014; Kallies et al., 2011; Lohoff et al., 2000; Samson et al., 2003; Taki et al., 2005; van Helden et al., 2015). Genetic deletion of Blimp-1 or Zeb2 also phenocopies impaired NK cell egress from the bone marrow and lymph nodes of T-bet-deficient mice (Jenne et al., 2009; Kallies et al., 2011; van Helden et al., 2015). The transcription factor Foxo1 negatively regulates late-stage NK cell maturation and promotes NK cell homing to lymph nodes, opposing the effects of T-bet (Deng et al., 2015). The foregoing

transcription factors are critical for the regulation of NK cell properties during later stages of development.

In addition to their role in NK cell maturation, the transcription factors T-bet, Eomes, GATA-3, IRF-2, and Aiolos act as redundant regulators of IFN-γ secretion that do not substantially affect NK cell cytotoxicity (Gordon et al., 2012; Holmes et al., 2014; Lohoff et al., 2000; Samson et al., 2003; Taki et al., 2005). T-bet and Eomes directly bind to the *Ifng*, *Prf1*, and *Gzmb* loci encoding IFN-γ, perforin, and granzyme B, respectively (Beima et al., 2006; Intlekofer et al., 2005; Pearce et al., 2003). In contrast, Foxo1 inhibits IFN-γ production and restrains the anti-tumor activity of NK cells (Deng et al., 2015). Other transcription factors are dispensable for NK development but regulate effector function following activation by pro-inflammatory cytokines (Sun, 2015). CEBP-γ, MITF, and MEF are required for NK cells to produce IFN-γ, express perforin, and mediate cytotoxicity (Ito et al., 2001; Kaisho et al., 1999; Lacorazza et al., 2002). Helios negatively regulates NK cell reactivity and becomes silenced in NK cells after maturation and activation (Narni-Mancinelli et al., 2012). Furthermore, Zbtb32 antagonizes the tumor suppressor Blimp-1 to facilitate proliferation of Ly49H$^+$ cells in the protective response against MCMV infection (Beaulieu et al., 2014). Finally, aryl hydrocarbon receptor (AhR) is critical for NK cell-mediated tumor immunosurveillance as well as IL-10 production required for the efficient resolution of *Toxoplasma gondii* infection (Shin et al., 2013; Wagage et al., 2014). Therefore, some transcription factors appear to control NK cell function without affecting other developmental processes.

Identification of distinct helper and cytotoxic/conventional NK cell lineages

NK cells are the founding members of the ILC family, but three additional ILC subsets, collectively known as helper ILCs, have recently been identified (Klose et al., 2014). The transcriptional programs and effector functions of helper ILCs closely resemble those of helper T cells, in contrast to cytotoxic NK cells that bear resemblance to cytotoxic T cells (Klose et al., 2014). Type 1 helper ILCs (hILC1s) express T-bet, produce Th1-associated cytokines (IFN-γ and TNF-α), and promote immunity against intracellular bacteria and parasites. Type 2 helper ILCs (hILC2s) express GATA-3, secrete Th2-associated cytokines (IL-4, IL-5, IL-9, and IL-13), and play important roles in anti-helminth immunity and allergic inflammation. Type 3 helper ILCs (hILC3s) express RORγt, produce Th17-associated cytokines (IL-17A, IL-17F, and IL-22), and are required for defense against extracellular bacteria (Artis and Spits, 2015; Diefenbach et al., 2014; Spits et al., 2013). Since the discovery of helper ILC subsets, ILCs have been categorized either as cytotoxic ILCs represented by conventional NK cells or as helper ILCs (hILC1s, hILC2s, hILC3s) that presumably have fewer cytotoxic capabilities (Klose et al., 2014)

Type 1 innate lymphocytes comprise two cell populations, type 1 helper innate lymphoid cells (hILC1s) and conventional NK (cNK) cells, which express the NK1.1 and NKp46 lineage antigens as well as the transcription factor T-bet. Both type 1 ILC populations also depend on IL-15 for development (Daussy et al., 2014; Klose et al., 2014). However, hILC1s and cNK cells can be distinguished according to expression of Eomes and numerous cell surface markers. $Eomes^{-}$ hILC1s constitutively express TRAIL and integrin CD49a, while $Eomes^{+}$ cNK cells are marked by expression of CD49b, Eomes, and diverse Ly49 receptors (Gordon et al., 2012; Marquardt et al., 2015; Peng et al., 2013; Takeda et al., 2005). hILC1s are sessile populations

(Peng et al., 2013; Sojka et al., 2014) whose maintenance and expansion occur locally in tissues under physiological and inflammatory conditions (Gasteiger et al., 2015). In contrast, cNK cells circulate through lymphoid and non-lymphoid tissues subsequent to their development in the bone marrow (Peng et al., 2013; Sojka et al., 2014). It has previously been suggested that hILC1s represent immature predecessors of cNK cells (Gordon et al., 2012; Takeda et al., 2005) based on evidence of a precursor-product relationship that has not been universally accepted (Daussy et al., 2014; Peng et al., 2013). However, recent studies proposed that hILC1s have common lineage origins with hILC2s and hILC3s, but represent a separate lineage from cNK cells (Constantinides et al., 2014; Klose et al., 2014).

Evidence for the distinct lineage origins of hILC1s and cNK cells came from identification of common helper innate lymphoid progenitors (CHILPs) that gave rise to all helper ILCs, but lacked cNK cell differentiation potential (Constantinides et al., 2014; Klose et al., 2014). Id2-expressing progenitors to all ILCs have previously been proposed (Spits et al., 2013) because the development of all ILC lineages is dependent on Id2 (Boos et al., 2007; Eberl et al., 2004; Monticelli et al., 2011; Moro et al., 2010; Vosshenrich and Di Santo, 2013; Yokota et al., 1999). One group identified CHILPs in the fetal liver and adult bone marrow that expressed Id2 and integrin $\alpha_4\beta_7$, but lacked expression of ILC lineage-defining transcription factors (Klose et al., 2014). $Id2^+$ progenitors expressed some markers of CLPs, but could be distinguished from CLPs by the absence of CD135 expression (Klose et al., 2014). Because $Id2^+$ CHILPs exclusively gave rise to helper ILCs, it was suggested that progenitors of cNK cells (NKPs) branch off from putative $Id2^-$ common innate lymphoid progenitors (CILPs) that are upstream of CHILPs (Klose et al., 2014). Despite descending from $Id2^-$ CILPs, cNK cells appear to upregulate Id2 expression

at later stages of development (Boos et al., 2007; Carotta et al., 2011; Fathman et al., 2011; Klose et al., 2014; Male et al., 2014).

The notion that hILC1s and cNK cells have disparate lineage origins was also supported by another study, which identified $\alpha_4\beta_7^+$ CHILPs in the fetal liver and adult bone marrow marked by expression of the transcription factor PLZF (Constantinides et al., 2014). PLZF has previously been associated with NKT development (Kovalovsky et al., 2008; Savage et al., 2008) and is not substantially expressed by mature ILC lineages (Constantinides et al., 2014). Like $Id2^+$ CHILPs, $PLZF^+$ CHILPs differentiated into helper ILCs, but failed to give rise to cNK cells (Constantinides et al., 2014; Klose et al., 2014). Because only a subpopulation of $Id2^+$ CHILPs expressed PLZF, $Id2^+$ $PLZF^+$ CHILPs might be descendants of $Id2^+$ $PLZF^-$ CHILPs (Klose et al., 2014). $PLZF^+$ CHILPs failed to give rise to lymphoid tissue inducer (LTi) cells unlike $PLZF^-$ CHILPs, suggesting that $PLZF^+$ CHILPs have a more restricted differentiation potential than $PLZF^-$ CHILPs (Constantinides et al., 2014; Klose et al., 2014). PLZF lineage tracing analyses revealed that helper ILC lineages were heavily labeled while cNK cells were labeled to a lesser extent, which supports the notion that hILC1s arise from $PLZF^+$ progenitors while cNK cells originate from $PLZF^-$ progenitors (Constantinides et al., 2014). In addition, PLZF is required for hILC1 development, but appears to be dispensable for cNK cell development (Constantinides et al., 2014).

The transcription factors NFIL3, TOX, and TCF-1 are necessary for the development of putative CILPs and CHILPs downstream of CLPs. As regulators of early ILC lineage specification, NFIL3, TOX, and TCF-1 are required for the development of all ILC lineages and are expressed

by all mature ILC subsets (Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015; Yu et al., 2014). In the setting of NFIL3, TOX, or TCF-1 deficiencies, analyses of ILC progenitors suggested that NFIL3, TOX, and TCF-1 appear to be upstream of Id2 and PLZF during early ILC development (Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015; Yu et al., 2014). NFIL3 and TCF-1, furthermore, regulate the emergence of NKPs, while TOX appears to be dispensable for NKP development (Aliahmad et al., 2010; Male et al., 2014; Seillet et al., 2014a). The molecular signals during early ILC hematopoiesis that underlie the eventual bifurcation of hILC1 and cNK cell lineages are currently being elucidated.

Parallels in the Development of Type 1 Innate Lymphocyte and T Cell Lineages

Despite having disparate lineage origins, hILC1s and cNK cells might remain inter-related after their developmental divergence. Evidence from T cell biology suggests that $CD4^+$ and $CD8^+$ T cell lineages have overlapping transcriptional networks and retain the potential for plasticity (Hirota et al., 2011; Lazarevic et al., 2013; Mucida et al., 2013; Reis et al., 2013; Tindemans et al., 2014). Transcriptome analyses have suggested that ILCs resemble T cells more closely than other blood lineages (Bezman et al., 2012). A similar dichotomy of helper and cytotoxic lineages occurs during both ILC and T cell development and subsequently, separate lineages appear to remain closely inter-related.

Like their adaptive $CD4^+$ and $CD8^+$ T cell counterparts, hILC1s and cNK cells are both dependent on the transcription factor GATA-3, albeit in different ways. GATA-3 function has been extensively studied in the context of $CD4^+$ T cell differentiation from a naïve to a committed Th2 cell fate (Tindemans et al., 2014). The development of hILC2s that mediate

type 2 immunity is also dependent on GATA-3 (Hoyler et al., 2012; Yagi et al., 2014). In addition to regulating Th2 and hILC2 differentiation, GATA-3 is expressed in $CD8^+$ T cells and has been implicated in their survival, activation, and proliferation (Wang et al., 2013). GATA-3 also functions in cNK cells to promote maturation, homeostasis, IFN-γ production in some tissues (Di Santo, 2006; Samson et al., 2003; Vosshenrich et al., 2006), even though GATA-3 is not essential for cNK cell development (Yagi et al., 2014). Moreover, GATA-3 promotes specification and survival of early T cell progenitors, as well as positive selection of $CD4^+$ T cells (Pai et al., 2003; Tindemans et al., 2014). In an analogous fashion, a role for GATA-3 has been proposed in the generation of early progenitors to helper ILC lineages (Constantinides et al., 2014; Klose et al., 2014; Yagi et al., 2014).

hILC1 and cNK cell lineages also appear to be inter-related because they are both dependent on T-bet, like $CD4^+$ and $CD8^+$ T cell lineages. T-bet was first recognized for its role in commitment to the type 1 T helper (Th1) cell fate and regulation of IFN-γ production by Th1 cells (Szabo et al., 2002). T-bet was also found to be critical for the development of type 1 helper ILCs or hILC1s (Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014). Furthermore, T-bet functions redundantly with Eomes to regulate $CD8^+$ T cell effector differentiation, IFN-γ production, and cytotoxicity (Intlekofer et al., 2008; Pearce et al., 2003). Evidence from previous studies suggests that there might be an analogous role for T-bet in cNK cell development and effector function (Gordon et al., 2012; Townsend et al., 2004).

Finally, the relationship between hILC1 and cNK cell lineages is of interest because there appears to be substantial plasticity between $CD4^+$ and $CD8^+$ T cell lineages, in addition to ILC1

and ILC3 lineages. $CD4^+$ T cells can acquire cytotoxic T cell attributes and lose helper properties (Mucida et al., 2013; Reis et al., 2013). Conversely, $CD8^+$ T cells can upregulate expression of helper genes at the expense of cytotoxic effector genes (Jenkinson et al., 2007). It has also been suggested that type 1 innate lymphocytes comprise a third cell population that is specified from the RORγt-dependent ILC3 lineage and acquires type 1 ILC properties under the control of T-bet (Klose et al., 2013; Sanos et al., 2009; Sciume et al., 2012). $NKp46^+$ "ex-RORγt" ILC1s are marked with a history of RORγt expression in lineage tracing analyses (Vonarbourg et al., 2010) and appeared to develop from RORγt-dependent ILC3s that upregulated T-bet and IFN-γ at the expense of RORγt and IL-17/IL-22 (Klose et al., 2013; Vonarbourg et al., 2010). A similar mechanism underlies plasticity from a Th17 to a Th1-like fate (Hirota et al., 2011). Type 1-polarizing cytokines, such as IL-12 and IL-18, favor the developmental transition from an ILC3 to an ILC1-like identity, while Type 3-polarizing cytokines, such as IL-23 and IL-1β, stabilize the ILC3 fate (Artis and Spits, 2015; Bernink et al., 2013; Klose et al., 2013; Vonarbourg et al., 2010). Antagonism of the transcription factor AhR induces an ILC3-to-ILC1 identity switch (Hughes et al., 2014), which is consistent with a role for AhR in the survival and function of ILC3s (Kiss et al., 2011; Lee et al., 2012). The degree of flexibility between type 1 innate lymphocyte lineages has yet to be determined and will ultimately contribute to our knowledge of how lymphocyte plasticity can be manipulated to curb the progression of inflammatory diseases (Diefenbach et al., 2014; Hirota et al., 2011; Vonarbourg et al., 2010).

In this thesis work, I characterized the relationship between type 1 innate lymphocyte lineages by examining the necessity and sufficiency of T-box factors for the maturation of hILC1s and cNK cells. I found that T-bet and Eomes are required for the development of all type 1 innate

lymphocytes across lymphoid and non-lymphoid tissues. I showed that Eomes is both necessary and sufficient to promote cNK cell development using novel mouse models. In addition, I integrated my findings with emerging transcription-based models of early ILC lineage specification to propose a refined model for the development of ILC lineages.

CHAPTER 2

T-BET AND EOMESODERMIN COORDINATE DEVELOPMENT OF ALL TYPE 1 INNATE LYMPHOCYTES

INTRODUCTION

Innate lymphoid cells are located at mucosal barriers in close proximity to epithelial surfaces, where they assume important roles in immunity, tissue repair, and inflammation (Artis and Spits, 2015). Type 1 innate lymphocytes (ILCs) express the NK1.1 and NKp46 lineage antigens as well as the transcription factor T-bet. Type 1 ILCs can be further subdivided into discrete populations based on expression of Eomesodermin (Eomes), a close homolog of T-bet (Daussy et al., 2014; Gordon et al., 2012). Eomes^{+} conventional NK (cNK) cells express integrin CD49b (DX5) and MHC-I specific Ly49 receptors and circulate through lymphoid and non-lymphoid organs. In contrast, Eomes^{-} helper innate lymphoid cells (hILC1s) express integrin CD49a and are anchored to their tissues and mucosal sites of origin (Cortez et al., 2014; Daussy et al., 2014; Fuchs et al., 2013; Gordon et al., 2012; Klose et al., 2014; Peng et al., 2013; Sojka et al., 2014). hILC1s and cNK cells have distinct cytokine profiles and differential expression of genes involved in trafficking and cytotoxicity, suggesting that they represent discrete type 1 ILC populations suited for unique functions (Daussy et al., 2014; Peng et al., 2013; Sojka et al., 2014).

hILC1s and cNK cells are thought to arise from separate progenitors (Constantinides et al., 2015; Constantinides et al., 2014; Klose et al., 2014) under the control of T-bet and Eomes, respectively (Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014).

Divergent lineage specification of hILC1s and cNK cells precedes expression of all type 1 ILC-associated markers, such as CD122, NK1.1, and NKp46 (Constantinides et al., 2014; Klose et al., 2014). The precise stage at which T-bet and Eomes play their essential roles during type 1 ILC development has yet to be determined. Our understanding of the molecular cues responsible for distinct outcomes of type 1 ILC development is further complicated by the possibility that tissue-specific populations might develop under the control of disparate transcriptional networks (Fuchs et al., 2013; Gordon et al., 2012; Klose et al., 2014; Seillet et al., 2014a; Sojka et al., 2014; Xu et al., 2015; Yagi et al., 2014; Yu et al., 2014). Using mice with germline and conditional deletions of T-bet and Eomes, we demonstrate that T-box factors act at the onset of type 1 ILC maturation to promote development of all lymphoid and non-lymphoid type 1 ILCs.

RESULTS AND DISCUSSION

Deletion of Eomes in NKp46^{+} ILCs blocks cNK cell development

Within various lymphoid and non-lymphoid tissues examined, we could identify hILC1s and cNK cells among lineage-negative, NK1.1^{+} NKp46^{+} cells based on reciprocal expression of integrins CD49a and CD49b, respectively (Figure 2.1A). We previously examined cNK cell development in mice with pan-hematopoietic deficiency of Eomes (Gordon et al., 2012). To determine if Eomes expression at the onset of ILC development is necessary for cNK cell differentiation, we intercrossed *Eomes*$^{flox/flox}$ (Intlekofer et al., 2008) and *NKp46-Cre*$^{+}$ (Narni-Mancinelli et al., 2011) mice. *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ mice had a substantial loss of CD49a^{-} DX5^{+} cNK cells (Figure 2.1B), which supports the prior suggestion that Eomes is genetically upstream of CD49b induction (Gordon et al., 2012). Remnant NK1.1^{+} NKp46^{+} cells in Eomes conditional knockout (cKO) mice were CD49a^{+} DX5^{-}, CD49a^{+} DX5^{+}, or CD49a^{-} DX5^{+} cells

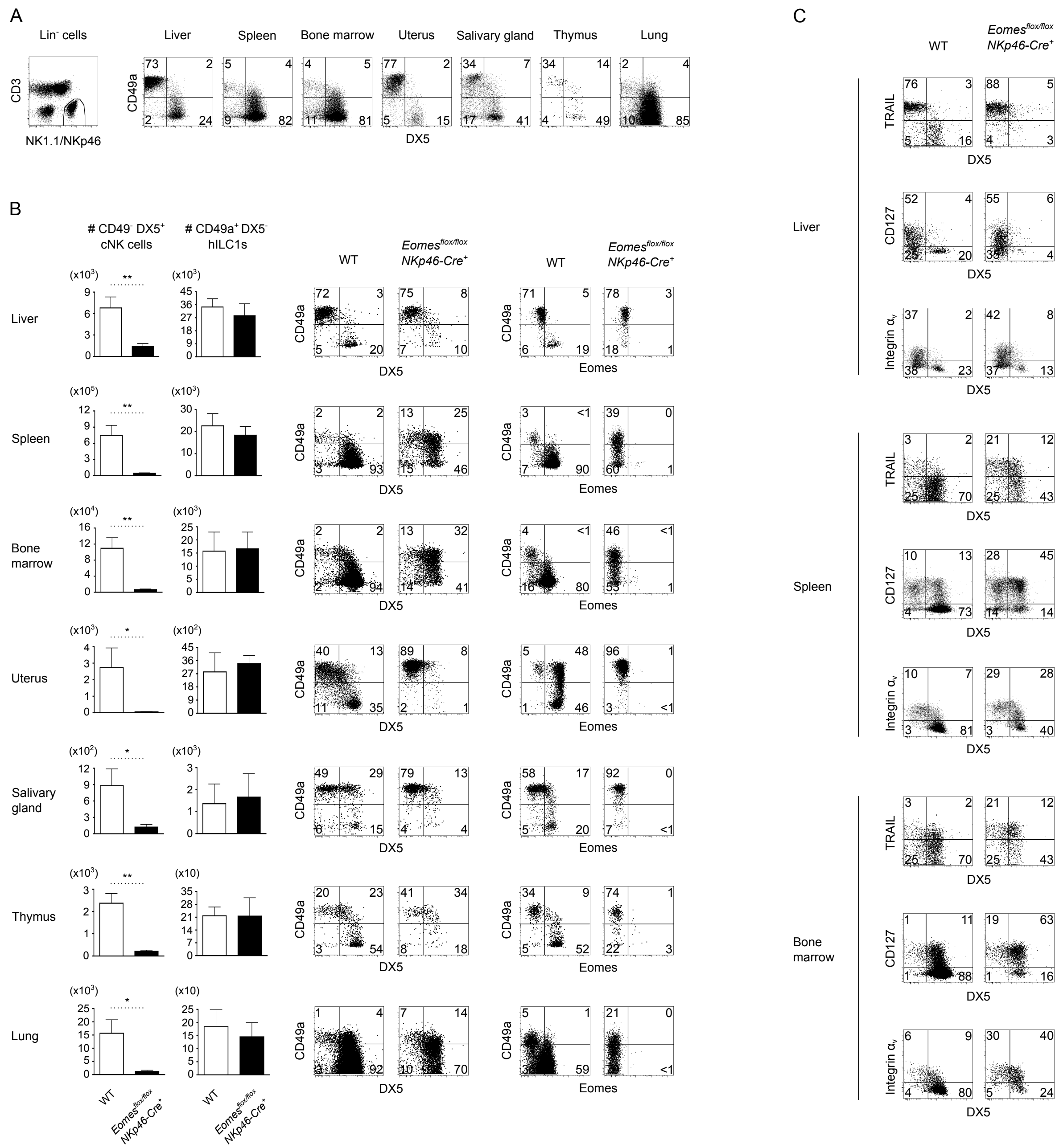


Figure 2.1. Loss of Eomes in NKp46$^+$ ILCs impairs cNK cell development.
(**A**) Representative flow cytometry of CD49a$^+$ DX5$^-$ hILC1 and CD49a$^-$ DX5$^+$ cNK cell subsets among type 1 ILCs [Lin (CD3, Gr-1, TER-119, CD19)$^-$ NK1.1$^+$ NKp46$^+$] from the indicated organs of wild-type (WT) mice (n=5 mice). The NK1.1/NKp46 gate is shown. (**B**) Flow cytometry of CD49a, CD49b (DX5), and Eomes expression by type 1 ILC subsets from the indicated organs of WT and *Eomes$^{flox/flox}$ NKp46-Cre$^+$* mice (n=4–5 mice per genotype). Absolute numbers of CD49a$^+$ DX5$^-$ hILC1s and CD49a$^-$ DX5$^+$ cNK cells are indicated. (**C**) Flow cytometry of TRAIL, CD127, and integrin α_v expression by type 1 ILCs from the indicated organs of WT and *Eomes$^{flox/flox}$ NKp46-Cre$^+$* mice (n=3 mice per genotype). Data are mean ± SEM representative of 3–5 independent experiments; *p<0.05, **p<0.01, ***p<0.001.

and the persistence of these cells did not appear to result from inefficient deletion of Eomes (Figure 2.1B). These findings are consistent with a role for Eomes upstream of CD49a repression, which is supported by prior evidence of direct binding of Eomes to the locus encoding CD49a (Teo et al., 2011). Examination of TRAIL, CD127, and integrin α_v confirmed that deletion of Eomes shifts the balance of type 1 ILCs from cNK cells to helper-like ILC1s (Figure 2.1C). The markers CD49a, TRAIL, CD127, and integrin α_v are highly expressed on hILC1s (Klose et al., 2014; Paust et al., 2010; Peng et al., 2013; Takeda et al., 2005), but have also been linked to immature cNK cells (Di Santo, 2006; Gasteiger et al., 2013; Gordon et al., 2012). Eomes appears to play its critical role in cNK cell maturation after the onset of NKp46 expression, resulting in repression of markers associated with an immature cNK cell or an alternative hILC1-like fate.

T-bet and Eomes regulate development of all type 1 ILCs

To test the contribution of T-bet to residual type 1 ILC development in Eomes cKO mice, we examined *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{-/-}$ mice. We used mice with a germline *Tbx21* deletion because *Tbx21*$^{flox/flox}$ *NKp46-Cre*$^{+}$ mice underwent substantial development or expansion of hILC1s that escaped *Tbx21* deletion (Figure 2.2A, B). *Tbx21*$^{flox/flox}$ *NKp46-Cre*$^{+}$ or T-bet conditional knockout (cKO) mice had a reduction in CD49a^{+} DX5^{-} Eomes^{-} hILC1s (Figure 2.2A). In contrast to *Tbx21*$^{-/-}$ mice with a germline deletion of T-bet, T-bet cKO mice had a substantial population of CD49a^{+} DX5^{-} Eomes^{-} hILC1s that expressed T-bet protein (Figure 2.2A). We examined whether remnant hILC1s in T-bet cKO mice underwent *Tbx21* deletion but contained residual T-bet protein or, alternatively, escaped *Tbx21* deletion. We used genomic PCR to analyze the status of the *Tbx21* locus in 3 cell populations sorted from the same

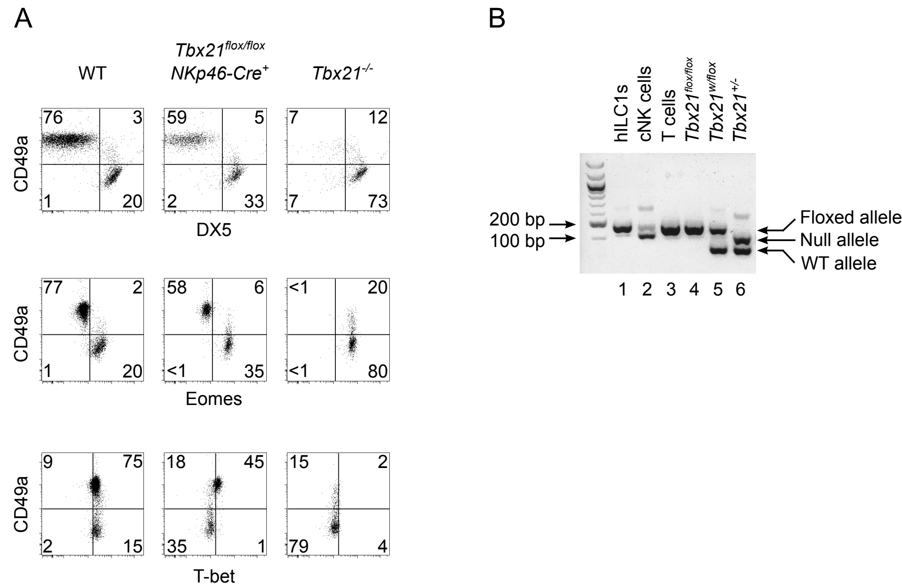


Figure 2.2. Incomplete loss of T-bet in NKp46$^+$ ILCs results in partial development or expansion of helper ILC1s. (**A**) Flow cytometry of hepatic NK1.1$^+$ NKp46$^+$ type 1 ILCs from WT, *Tbx21*$^{flox/flox}$ *NKp46-Cre*$^+$ (T-bet conditional KO or cKO), and *Tbx21*$^{-/-}$ mice (n=3 mice per genotype). In contrast to *Tbx21*$^{-/-}$ mice with a germline deletion of T-bet, T-bet cKO mice had a substantial population of CD49a$^+$ DX5$^-$ Eomes$^-$ hILC1s that expressed T-bet protein. (**B**) Genomic PCR analysis of the status of the *Tbx21* locus in 3 cell populations sorted from the same *Tbx21*$^{flox/flox}$ *NKp46-Cre*$^+$ mouse (n=2 mice): CD49a$^+$ DX5$^-$ hILC1s (lane 1), CD49a$^-$ DX5$^+$ cNK cells (lane 2), and CD3$^+$ T cells (lane 3). The following 3 primers were used to amplify WT and mutant *Tbx21* loci: (1) TAT GAT TAC ACT GCA GCT GTC TTC AG, (2) CAG GAA TGG GAA CAT TCG CCT GTG, and (3) CTC TGC CTC CCA TCT CTT AGG AGC. DNA samples from the sorted populations (lanes 1–3) were compared to DNA samples from the skin of mice that have WT, null, or floxed *Tbx21* alleles (lanes 4–6). Data are representative of 2–3 independent experiments.

Tbx21$^{flox/flox}$ *NKp46-Cre*$^{+}$ mouse (Figure 2.2B): CD49a^{+} DX5^{-} hILC1s, CD49a^{-} DX5^{+} cNK cells, and CD3^{+} T cells. Comparison with control skin samples from mice that carry wild-type (WT), null, or floxed *Tbx21* alleles led us to conclude that the majority of hILC1s and a minor subset of cNK cells in T-bet cKO mice escaped *Tbx21* deletion (Figure 2.2B). NKp46-Cre-driven *Tbx21* deletion did not occur in T cells (Figure 2.2B), suggesting that there did not appear to be off-target Cre recombinase activity in T-bet cKO mice.

Compound deficiency of T-bet and Eomes resulted in almost complete elimination of the type 1 ILC compartment in all tissues examined (Figure 2.3). NK1.1^{+} NKp46^{+} type 1 ILCs in the liver, spleen, and lung depended on the semi-redundant function of T-bet and Eomes, while type 1 ILCs in the bone marrow and thymus primarily required Eomes (Figure 2.3). In contrast, type 1 ILCs in the salivary gland and uterus seemed to withstand the loss of one but not both factors (Figure 2.3). Our results, in conjunction with previous findings (Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014), suggest that both T-bet and Eomes are required in order to develop the spectrum of lymphoid and non-lymphoid type 1 ILCs.

T-bet and Eomes have redundant roles in cNK cell and helper ILC1 development

One might postulate that T-bet and Eomes account for the totality of type 1 ILCs because T-bet and Eomes play opposing roles in the development of hILC1s and cNK cells, respectively. However, previous studies support the possibility that T-bet and Eomes might have redundant functions in cNK cell development (Gordon et al., 2012; Townsend et al., 2004), in an analogous manner to their roles in CD8^{+} effector T cell differentiation (Intlekofer et al., 2008; Pearce et al., 2003). In support of this notion, a recent study reported that T-bet expression rescued cNK

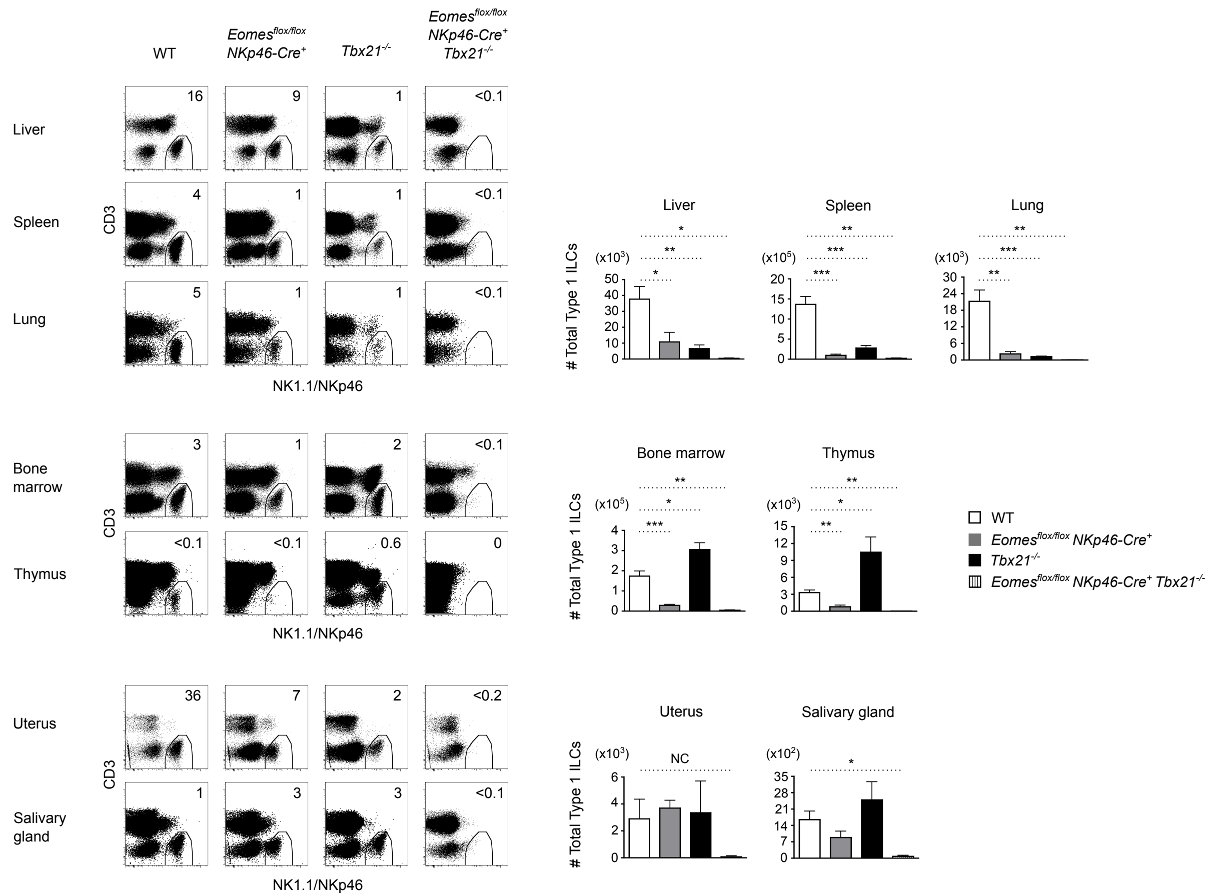


Figure 2.3. T-bet and Eomes coordinate development of all lymphoid and non-lymphoid type 1 ILCs. Representative flow cytometry of NK1.1^{+} NKp46^{+} type 1 ILCs from the indicated organs of WT, *Eomes$^{flox/flox}$ NKp46-Cre^{+}*, *Tbx21$^{-/-}$*, and *Eomes$^{flox/flox}$ NKp46-Cre^{+} Tbx21$^{-/-}$* mice (n=3–5 mice per genotype). Absolute numbers of type 1 ILCs are indicated. Statistics were not calculated (NC) for the difference between WT and *Eomes$^{flox/flox}$ NKp46-Cre^{+} Tbx21$^{-/-}$* uterus because the *Eomes$^{flox/flox}$ NKp46-Cre^{+} Tbx21$^{-/-}$* uterus group represents the average of 2 mice. All other data are mean ± SEM representative of 3–5 independent experiments; *p<0.05, **p<0.01, ***p<0.001.

cell development from progenitors with a germline deletion of NFIL3 (Male et al., 2014), a transcription factor that specifies the earliest stages of ILC development.

Examination of the type 1 ILC compartment in *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ (Eomes cKO) mice revealed a substantial but, nonetheless, incomplete loss of cNK cells (Figure 2.1B). Among the remnant cNK cells in Eomes cKO mice, we observed helper-like subpopulations that expressed CD49a, TRAIL, CD127, and integrin α_v at higher frequencies than WT cNK cells (Figure 2.1B, C). To determine if residual cNK cell development in Eomes cKO mice is dependent on T-bet, we examined *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ alongside *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{-/-}$ mice. Our analyses revealed that *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ mice contained fewer cNK cells when intercrossed to T-bet-deficient mice, suggesting that T-bet might partially substitute for Eomes during cNK cell development (Figure 2.4A). We investigated the contribution of T-bet to cNK cell development primarily using comparisons of *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ and *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{-/-}$ mice rather than analyses of *Tbx21*$^{-/-}$ mice. Because T-bet regulates cNK cell egress from the bone marrow and lymph nodes (Jenne et al., 2009), the altered distribution of type 1 ILCs in the tissues of *Tbx21*$^{-/-}$ mice (Figure 2.3) could be the result of a cNK cell trafficking phenotype, rather than a developmental phenotype. By illustrating a role for T-bet in the Eomes-independent development of cNK cells, our findings suggest that T-bet and Eomes serve as semi-redundant regulators of cNK cell development. T-bet and Eomes, therefore, do not simply play opposing roles during type 1 ILC development.

If T-bet and Eomes could substitute for each other's functions during type 1 ILC development, then it is possible that Eomes might also contribute to residual hILC1 development in *Tbx21*$^{-/-}$

Figure 2.4. T-bet and Eomes have redundant roles in cNK cell and helper ILC1 differentiation. (**A**) Flow cytometry of DX5^{+} (CD49a^{+} DX5^{+} and CD49a$^{–}$ DX5^{+}) subsets among NK1.1^{+} NKp46^{+} type 1 ILCs from the indicated organs of WT, *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$, and *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{–/–}$ mice (n=3–5 mice per genotype). Absolute numbers of cNK cells are indicated. (**B**) Flow cytometry of CD49a^{+} DX5$^{–}$ hILC1 subsets from the indicated organs of WT, *Tbx21*$^{–/–}$, and *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{–/–}$ mice (n=3–5 mice per genotype). The *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{–/–}$ uterus group represents the average of 2 mice. Absolute numbers of hILC1s are indicated. (**C**) Flow cytometry of CD127, TRAIL, Ly49C/I, CD122, Eomes, and Ly49D/G2/H expression by hILC1s from the indicated organs of WT mice (n=2–3 mice). Data are mean ± SEM representative of 2–5 independent experiments; *p<0.05, **p<0.01, ***p<0.001.

mice. Our analyses of *Tbx21*$^{-/-}$ mice (Figure 2.4A, B) corroborated the previous suggestions that hILC1s from the uterus develop independently of T-bet, while hILC1s from the liver and other tissues require T-bet for development (Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014). Examination of *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ mice illustrated that hILC1 development withstands the loss of Eomes across all organs (Figure 2.1B). Our analyses of *Tbx21*$^{-/-}$ alongside *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{-/-}$ mice, furthermore, illustrated that compound deficiency of T-bet and Eomes resulted in substantial impairment in uterine hILC1 development (Figure 2.4B). Uterine hILC1 development, therefore, is redundantly regulated by T-bet and Eomes (Figure 2.4B), in contrast to hepatic hILC1 development which is primarily dependent on T-bet (Figure 2.4A). Consistent with the disparity in their developmental requirements, hILC1s that differentiate in the hepatic milieu are phenotypically distinct from hILC1s that develop in the uterine microenvironment (Figure 2.4C). Relative to hepatic hILC1s, uterine hILC1s had lower expression of several hILC1-associated markers (Gordon et al., 2012; Klose et al., 2014; Takeda et al., 2005), such as CD127, TRAIL, and Ly49C/I (Figure 2.4C). Uterine hILC1s also had abundant expression of CD122, Eomes, and Ly49 receptors, in contrast to their hepatic counterparts (Figure 2.4C). Our data suggest that Eomes might partially substitute for T-bet during uterine hILC1 development insofar as *Tbx21*$^{-/-}$ mice contain fewer hILC1s when intercrossed to Eomes-deficient mice.

This study demonstrates that formation of the entire lymphoid and non-lymphoid type 1 ILC compartment requires the semi-redundant action of T-bet and Eomes. Type 1 ILCs in distinct environments appear to be inter-related because of their dependence on T-bet and Eomes, even though there is evidence to suggest that their development is orchestrated by tissue-specific

transcription factor networks (Fuchs et al., 2013; Gordon et al., 2012; Klose et al., 2014; Seillet et al., 2014a; Sojka et al., 2014; Xu et al., 2015; Yagi et al., 2014; Yu et al., 2014). Furthermore, Eomes plays its essential role in cNK cell differentiation after the onset of NKp46 expression in developing type 1 ILCs across all organs. T-bet and Eomes have unique non-redundant functions that are critical for the development of hILC1s and cNK cells, respectively. The structural homology between T-bet and Eomes, however, appears to enable each factor to partially substitute for the other's function during development. T-bet partially supports cNK cell development in the absence of Eomes. Conversely, Eomes contributes to hILC1 development in the event of T-bet's absence. The notion that T-bet and Eomes have redundant roles in the differentiation of both lineages argues against a previous model in which hILC1s and cNK cells are regarded as mutually exclusive T-bet-dependent and Eomes-dependent lineages, respectively (Daussy et al., 2014). Our study suggests that hILC1 and cNK cell lineages might remain inter-related to a greater degree than is currently appreciated.

MATERIALS AND METHODS

Mice

Mice were housed in specific pathogen-free conditions and used in accordance with the Columbia University Institutional Animal Care and Use Committee's guidelines. C57BL/6 *Eomes*$^{flox/flox}$, *Tbx21*$^{flox/flox}$, *Tbx21*$^{-/-}$ (Intlekofer et al., 2008; Intlekofer et al., 2005), and *NKp46-Cre*$^{+}$ (Narni-Mancinelli et al., 2011) mice were generated as previously described. They were bred locally to obtain the compound genotypes *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$, *Tbx21*$^{flox/flox}$ *NKp46-Cre*$^{+}$, and *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{-/-}$. Adult mice (4-16 weeks of age) were used for analyses.

Lymphocyte isolation and flow cytometry

Livers were dissected and thoroughly perfused with PBS. Single-cell suspensions were prepared from freshly harvested liver, spleen, bone marrow, and thymus after mechanical dissociation and passage through 70 μm cell strainers. Livers were subsequently resuspended in 40% Percoll at room temperature (GE Healthcare) and centrifuged over 60% Percoll at 2000 rpm for 20 minutes. Liver lymphocytes were isolated at the interphase of the Percoll gradient. Red blood cells in spleen, bone marrow, and thymus suspensions were lysed using ACK lysing buffer (ThermoFisher Scientific).

Freshly harvested uterus, salivary glands, and lungs were cut into small pieces and digested in the presence of DNase I and either collagenase D or liberase TL (Roche Life Science) for 1 hour, as suggested in previous studies (Cortez et al., 2014; Sojka et al., 2014). Trypsin inhibitor (Roche Life Science) was additionally incorporated into lung digestion reactions. Following digestion,

single-cell suspensions were prepared from uterus, salivary gland, and lung tissues after mechanical dissociation and passage through 70 µm cell strainers. Red blood cells in salivary gland and lung suspensions were lysed using ACK lysing buffer (ThermoFisher Scientific).

Cells were stained with a LIVE/DEAD fixable dead cell stain kit (Invitrogen) and blocked with antibodies against CD16/CD32 (BD) prior to staining with cell surface antibodies for CD3, CD122, integrin α_v, Ly49C/I, Ly49G2, Ly49H, NKp46, and TRAIL (eBioscience); CD19, CD49a, CD49b, CD127, Ly49D, Ly-6G/Ly-6C (Gr-1), NK1.1, and TER-119 (BD). Cells were treated with the eBioscience cytofix/cytoperm kit prior to staining with antibodies for the transcription factors T-bet (eBioscience) and Eomes (eBioscience). Cells were analyzed on an LSRFortessa coupled to FACSDiva software. Data were analyzed with FlowJo software (Treestar).

Statistical analysis

Statistics were calculated using Prism (GraphPad Software). Differences between groups were quantified using the unpaired *t*-test. Statistical significance was reached at $p < 0.05$.

CHAPTER 3

EOMESODERMIN, NOT T-BET, ACTS AS A DETERMINANT OF TYPE 1 INNATE LYMPHOCYTE FATE

INTRODUCTION

The absence or presence of Eomes after the onset of NKp46 expression results in distinct outcomes of type 1 ILC development. The unique functions of T-bet and Eomes are essential for Eomes^{-} hILC1 versus Eomes^{+} cNK cell maturation. However, the structural homology between T-bet and Eomes also permits each factor to partially compensate for the other's function. hILC1s and cNK cells appear to overlap in their dependency on T-bet and Eomes, which suggests that type 1 ILC lineages might remain inter-related subsequent to their divergence from separate precursors (Constantinides et al., 2014; Klose et al., 2014). In support of this notion, global gene expression analyses revealed that hILC1s and cNK cells are transcriptionally related and have few characteristic transcripts relative to other ILC subsets (Robinette et al., 2015). There might also be a significant degree of plasticity between hILC1s and cNK cells insofar as each lineage can acquire characteristics of the other lineage (Gordon et al., 2012; Takeda et al., 2005). Using novel transgenic mouse lines, we examined the sufficiency of T-bet and Eomes to determine the outcome of type 1 ILC maturation. Our findings lead us to the conclusion that Eomes acts as a key determinant of cNK cell versus hILC1 maturation following lineage specification.

RESULTS AND DISCUSSION

Deletion of Eomes directs cNK cells toward a helper ILC1-like fate

Temporal deletion of Eomes from cNK cells has previously been suggested to unveil latent hILC1-like characteristics (Gordon et al., 2012). hILC1s are now defined by expression of integrin CD49a (Peng et al., 2013), in addition to expression of TRAIL and absence of integrin CD49b (Gordon et al., 2012; Takeda et al., 2005). Therefore, we re-examined whether temporal deletion of Eomes induces hILC1-like properties among cNK cells using the most current definition of hILC1s. Splenic cNK cells with floxed *Eomes* alleles were treated directly ex vivo with media alone (sham) or with Cre recombinase fused to the HIV TAT protein transduction domain (Wadia et al., 2004), also known as TAT-Cre. Sham-treated or TAT-Cre-treated cells were transferred into separate lymphopenic $Il2rg^{-/-}$ $Rag2^{-/-}$ recipients.

From recipients of sham-treated cells, we recovered a major $NK1.1^{+}$ $NKp46^{+}$ population of $Eomes^{+}$ $DX5^{+}$ cNK cells that expressed diverse Ly49 receptors and closely resembled the donor population (Figure 3.1A). From recipients of TAT-Cre-treated cells, we additionally recovered an $NK1.1^{+}$ $NKp46^{+}$ population of $Eomes^{-}$ cells that expressed multiple Ly49 receptors, which probably represented former cNK cells that underwent Cre-mediated deletion of their *Eomes* alleles (Figure 3.1A). The stability of Ly49 receptor expression among acutely Eomes-deficient $NK1.1^{+}$ $NKp46^{+}$ type 1 ILCs suggested that Eomes is dispensable for the maintenance of Ly49 receptors (Figure 3.1A), even though it is required for Ly49 receptor acquisition (Gordon et al., 2012). We also demonstrated that acutely Eomes-deficient type 1 ILCs, sorted according to expression of Ly49D and Ly49H receptors, exhibited heritable maintenance of their respective Ly49 receptor profiles (Figure 3.1B). Although Ly49 receptor expression was retained after

temporal deletion of Eomes, other characteristics of cNK cells appeared to be lost. A substantial proportion of NK1.1^{+} NKp46^{+} type 1 ILCs that lost their *Eomes* alleles upregulated expression of CD49a and downregulated expression of CD49b (DX5) and CD122, in contrast to cells that retained their *Eomes* alleles (Figure 3.1A). Temporal deletion of Eomes did not result in upregulation of other hILC1-associated markers, such as CD127 and integrin α_v (Figure 3.1A). These findings suggest that temporal deletion of Eomes from cNK cells allows them to more closely resemble hILC1s. However, loss of Eomes is not sufficient to convert all attributes of cNK cells to those of hILC1s.

Because T-bet is a requirement for hILC1 development (Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014), we investigated whether deletion of Eomes triggers plasticity from a cNK cell to an hILC1-like identity in a T-bet-dependent manner. We temporally deleted Eomes from splenic cNK cells with floxed *Eomes* alleles on T-bet sufficient (*Tbx21*$^{+/+}$) and T-bet deficient (*Tbx21*$^{-/-}$) backgrounds. Sham-treated or TAT-Cre-treated samples from T-bet-sufficient and T-bet-deficient groups were transferred into separate *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ recipients. *Tbx21*$^{+/+}$ cNK cells that lost their *Eomes* alleles maintained expression of the type 1 ILC-associated NK1.1 antigen, upregulated expression of CD49a, and downregulated expression of CD49b (Figure 3.1C), as discussed above. However, *Tbx21*$^{-/-}$ cNK cells with temporally deleted *Eomes* alleles failed to upregulate CD49a expression and additionally downregulated expression of other type 1 ILC-associated antigens, including NK1.1 and CD49b (Figure 3.1C). *Tbx21*$^{-/-}$ cells that were acutely Eomes-deficient maintained expression of CD122, albeit at somewhat lower levels (Figure 3.1C). These results suggest that temporal deletion of Eomes induces

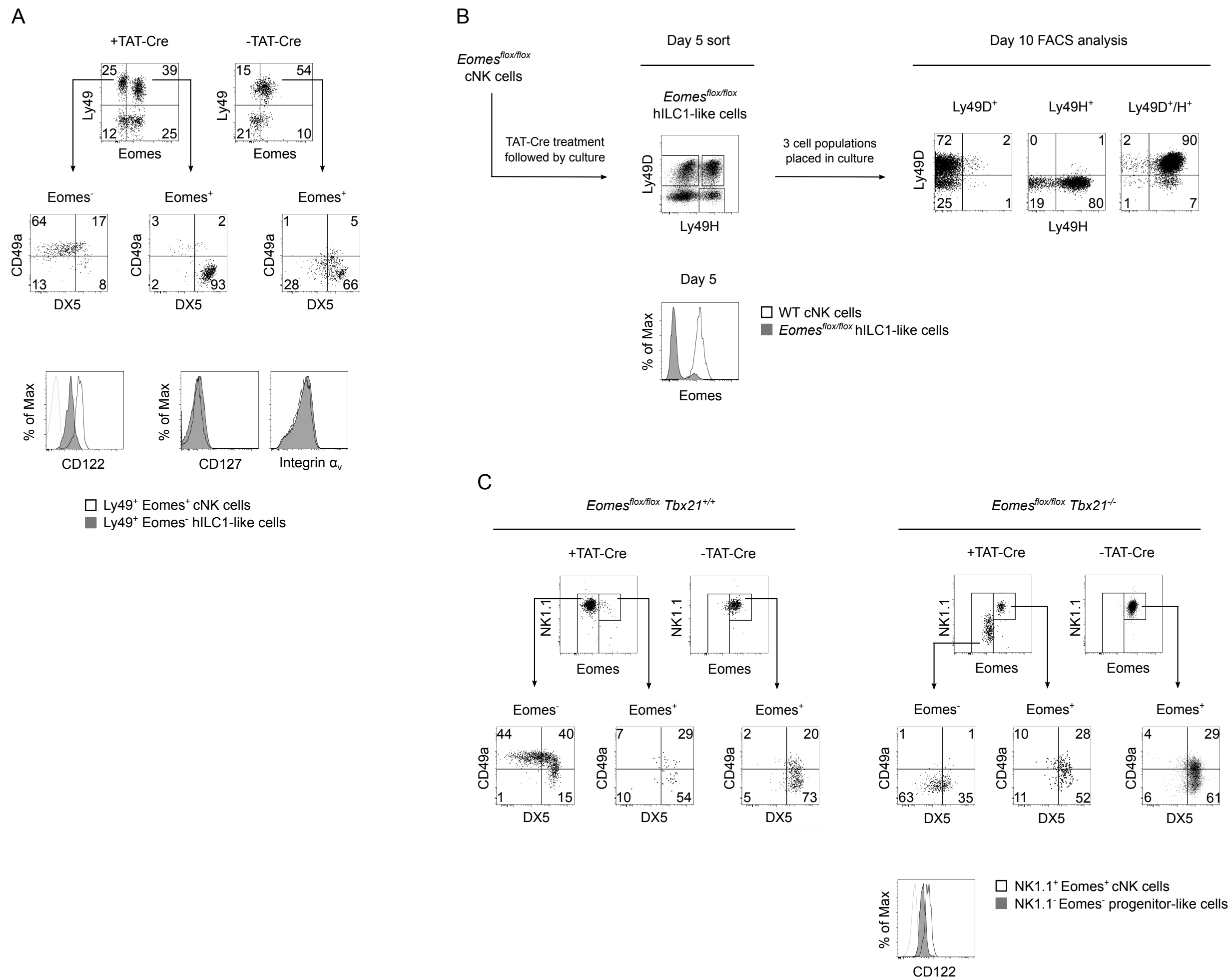


Figure 3.1. Deletion of Eomes directs cNK cells toward a helper ILC1-like fate in a T-bet-dependent manner. (**A-C**) Flow cytometry of splenic cNK cells with floxed *Eomes* alleles that were treated with TAT-Cre or sham-treated directly ex vivo. Cells from both groups were transferred into separate *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ recipients (n=3 recipients per group) or cultured in the presence of rIL-2. (A) Expression of Ly49D/G2/H receptors by NK1.1^{+} NKp46^{+} cells remained stable after temporal deletion of *Eomes* alleles. Ly49^{+} cells that maintained or lost Eomes protein expression were analyzed for expression of CD49a, CD49b (DX5), and CD122. Expression of CD127 and integrin α_v was examined in a subsequent analysis. (B) cNK cells treated with TAT-Cre were placed in culture. On day 5, NK1.1^{+} NKp46^{+} cells lost substantial Eomes protein expression, suggesting that their *Eomes* alleles were temporally deleted. Helper-like NK1.1^{+} NKp46^{+} cells that expressed TRAIL and lacked CD49b were sorted into 3 cell populations that expressed Ly49D, Ly49H, or both. The sorted populations were placed in culture until day 10. (C) Flow cytometry of *Tbx21*$^{+/+}$ and *Tbx21*$^{-/-}$ splenic cNK cells after TAT-Cre or sham treatment. Ly49^{+} cells that maintained or lost Eomes protein expression were analyzed for expression of NK1.1, CD49a, CD49b, and CD122. (A, C) Negative controls (light gray) are indicated in histogram plots to illustrate downregulation but not complete absence of CD122 expression. Data are representative of 2–4 independent experiments.

T-bet-dependent plasticity from a cNK cell to an hILC1-like fate. In the absence of T-bet, cNK cells appear to dedifferentiate into a progenitor-like state.

Helper ILC1s displaced to ectopic sites do not rapidly upregulate Eomes

It has also been suggested $Eomes^{-}$ hILC1s can de-repress their Eomes expression and transition to $Eomes^{+}$ cNK cells (Gordon et al., 2012; Takeda et al., 2005). However, the notion that hILC1s can acquire Eomes expression and cNK cell properties has not been universally accepted in the field (Daussy et al., 2014; Klose et al., 2014; Peng et al., 2013). Examination of hILC1 and cNK cell subsets from the liver, spleen, and bone marrow suggested that the hepatic milieu stabilizes the hILC1 lineage by promoting high-level expression of T-bet and PLZF (Figure 3.2A), transcription factors required for the development of hILC1s (Constantinides et al., 2014; Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014). Displacement of $NK1.1^{+}$ $NKp46^{+}$ type 1 ILCs from the liver to the spleen and bone marrow resulted in repression of T-bet and PLZF (Figure 3.2B), which could potentially favor development of $Eomes^{+}$ cNK cells (Constantinides et al., 2014; Daussy et al., 2014).

To determine whether hILC1s can convert to cNK cells, we seeded liver-derived hILC1s to ectopic sites where hILC1s could potentially de-repress their Eomes expression. We transferred CD45.1 donor hILC1s into CD45.2 $Il2rg^{-/-}$ $Rag2^{-/-}$ recipients whose lymphopenic spleen and bone marrow compartments could accommodate the entry of displaced hILC1s without significant competition from other lymphocytes. We employed a short time point of 24 hours for adoptive transfer experiments to minimize the possibility of cNK cells arising from selective proliferation of a contaminant rather than from rapid conversion from hILC1s. From CD45.2

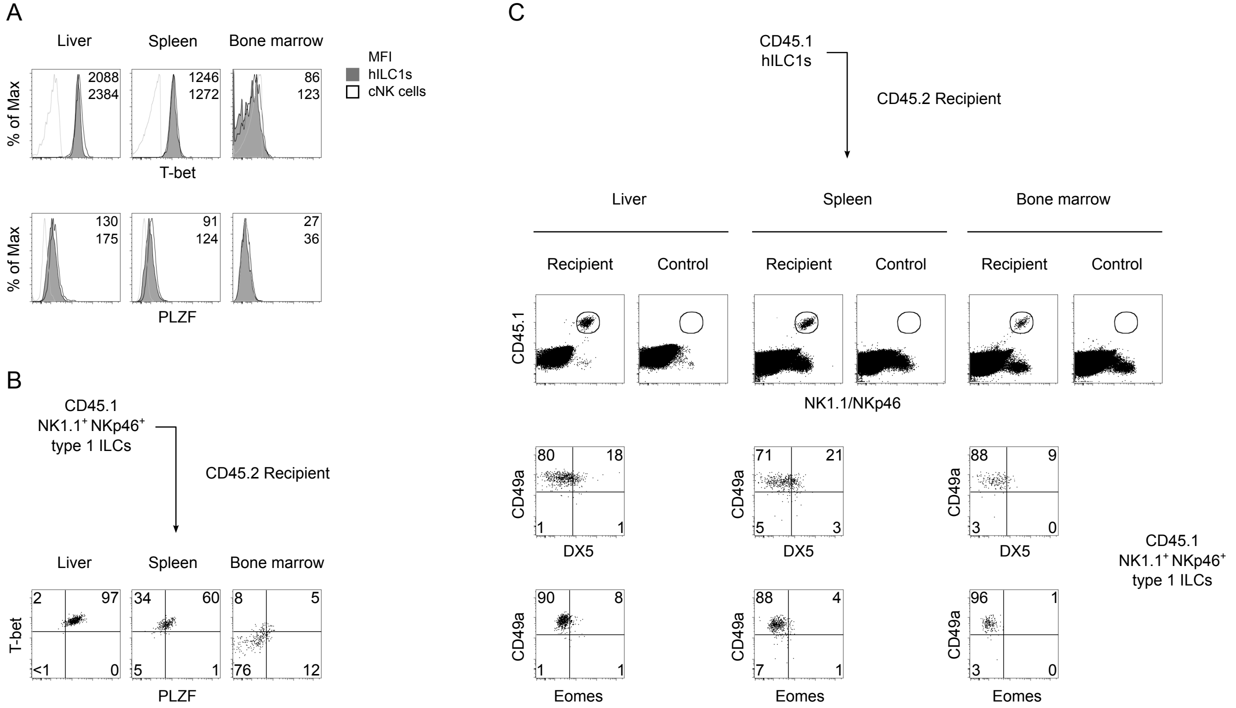


Figure 3.2. Helper ILC1s displaced to extra-hepatic sites do not rapidly upregulate Eomes. (**A**) Flow cytometry of T-bet and PLZF expression by CD49a^{+} DX5^{-} hILC1s and CD49a^{-} DX5^{+} cNK cells from the indicated organs of WT mice (n=3 mice). (**B**) CD45.1 NK1.1^{+} NKp46^{+} type 1 ILCs derived from liver were transferred into CD45.2 *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ recipients. Flow cytometry of T-bet and PLZF expression by CD45.1 NK1.1^{+} NKp46^{+} donor cells in CD45.2 recipients (n=3 recipient mice). (**C**) CD45.1 hILC1s derived from liver were transferred into CD45.2 *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ recipients. Flow cytometry of CD49a, CD49b, and Eomes expression by CD45.1 NK1.1^{+} NKp46^{+} donor cells in CD45.2 recipients (n=2 recipient mice). Control CD45.2 *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ mice that were not recipients of adoptive transfer were analyzed alongside recipients of CD45.1 hILC1s. Data are representative of 2–3 independent experiments.

Il2rg$^{-/-}$ *Rag2*$^{-/-}$ recipients, we retrieved a population of CD45.1 NK1.1^{+} NKp46^{+} type 1 ILCs that expressed CD49a but lacked substantial expression of CD49b and Eomes across all organs (Figure 3.2C). Our findings illustrate that hILC1s displaced from the liver do not appear to rapidly upregulate Eomes expression and convert to cNK cells.

Transgenic Eomes induces cNK cell attributes among type 1 ILCs

Our study, in conjunction with other reports (Daussy et al., 2014; Klose et al., 2014; Peng et al., 2013), suggests that endogenous Eomes expression in hILC1s cannot be rapidly elicited in vivo or in vitro. We generated a novel transgenic mouse model in which Eomes codons are expressed according to *Tbx21* locus control elements, allowing Eomes expression to be enforced in T-bet-dependent hILC1s (Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014). Because Eomes acts at the NKp46-expressing stage to promote cNK cell development (see Chapter 2, Figure 2.1), we hypothesized that enforced Eomes expression at the onset of hILC1 lineage maturation would be sufficient to redirect hILC1s into a cNK cell identity. T-bet is not expressed at substantial levels until type 1 ILCs acquire the lineage antigens NK1.1 and NKp46 (Gordon et al., 2012; Townsend et al., 2004), which allows us to study the effect of transgenic Eomes expression following divergent lineage specification from NK1.1^{-}NKp46^{-} progenitors (Constantinides et al., 2014; Klose et al., 2014).

We verified that transgenic Eomes is expressed when and where T-bet is expressed by examining the pattern of Eomes expression in 3 cell populations that ordinarily express T-bet but not Eomes: neonatal type 1 ILCs, NK1.1^{+} T cells, and developing Th1 cells (Gordon et al., 2012; Lazarevic et al., 2013). In each case, transgenic cells expressed Eomes in proportion to T-bet

(Figure 3.3A). Transgenic Eomes did not affect the overall development of neonatal type 1 ILCs or $CD4^+$ T cells, but it resulted in moderate reduction of hepatic $NK1.1^+$ T cells (Figure 3.3A). Analyses of type 1 ILCs revealed that transgenic Eomes perturbed the normal spatial and temporal pattern of hILC1 development. Transgenic Eomes caused a shift in frequency and absolute numbers toward CD49b (DX5) expression among most lineage-negative, $NK1.1^+$ $NKp46^+$ type 1 ILCs in lymphoid and non-lymphoid tissues (Figure 3.3B, C). In addition to the shift in adult ILC subsets, transgenic neonates exhibited precocious development of $DX5^+$ type 1 ILCs, compared to the minor population evident in WT neonates (Figure 3.3D). Finally, $Eomes^-$ hILC1s have been suggested to express higher levels of TNF-α than $Eomes^+$ cNK cells (Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014). Consistent with the observed shift in integrin expression, we found that transgenic Eomes attenuated TNF-α expression by hepatic type 1 ILCs (Figure 3.3E). Eomes expression, thus, appears sufficient to redirect hILC1s into a more cNK cell-like fate.

Transgenic Eomes is repressive to T-bet expression by type 1 ILCs

Our findings revealed that transgenic Eomes shifted the balance of type 1 ILCs toward the cNK cell fate. We investigated whether the reduction in hILC1s evident in transgenic mice might be attributable to the repression of T-bet by transgenic Eomes. T-bet is a repressor of Eomes expression (Daussy et al., 2014; Gordon et al., 2012) and it is possible that Eomes might also serve as a negative regulator of T-bet expression. Examination of T-bet and Eomes levels revealed that transgenic type 1 ILC subsets had repressed T-bet expression (Figure 3.4A), suggesting that repression of T-bet by transgenic Eomes might explain the phenotypic shift in type 1 ILCs (Figure 3.3B) and the reduction in $NK1.1^+$ T cells (Figure 3.3A). The repressive

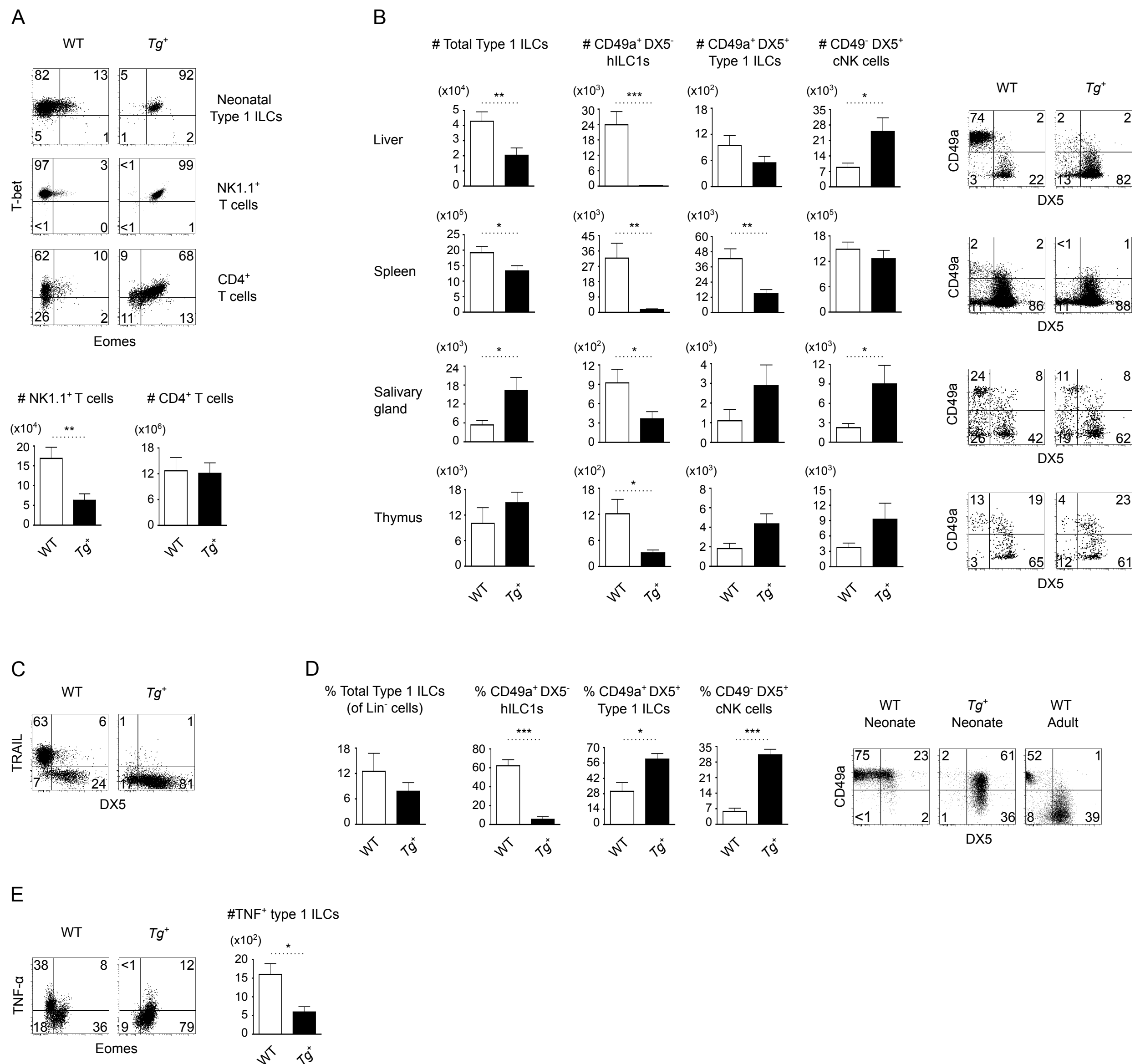
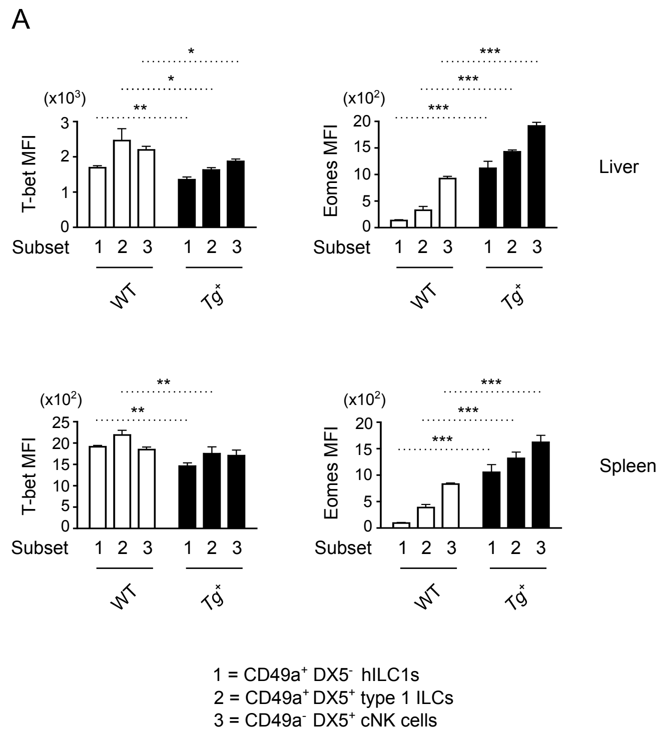


Figure 3.3. Eomes induces cNK cell properties among type 1 ILCs. (**A**) Transgenic (*Tg*$^+$) Eomes expression in 3 cell populations (from at least 3 mice per genotype) that usually express T-bet but not Eomes: NK1.1$^+$ NKp46$^+$ type 1 ILCs from 3-day old neonatal livers; NK1.1$^+$ CD3$^+$ T cells from adult livers; and stimulated CD4$^+$ T cells from adult spleens. Absolute numbers of NK1.1$^+$ T cells and CD4$^+$ T cells are indicated. (**B**) Flow cytometry and absolute numbers of CD49a$^+$ DX5$^-$, CD49a$^+$ DX5$^+$, and CD49a$^-$ DX5$^+$ type 1 ILC subsets from the indicated organs of WT and *Tg*$^+$ mice (n=4–5 mice per genotype). (**C**) Flow cytometry of TRAIL and CD49b expression by hepatic type 1 ILCs from WT and *Tg*$^+$ mice (n=3 mice per genotype). (**D**) Flow cytometry of hepatic type 1 ILC subsets from WT neonates, *Tg*$^+$ neonates, and WT adult mice (n=4 mice per genotype). Frequencies of neonatal type 1 ILC subsets are shown. (**E**) Flow cytometry of TNF-α expression by stimulated hepatic type 1 ILCs from WT and *Tg*$^+$ mice (n=4 mice per genotype). Data were obtained from mice that have 1 copy of the transgene (*Tg*$^+$). Data are mean ± SEM representative of 2–5 independent analyses; *p<0.05, **p<0.01, ***p<0.001.



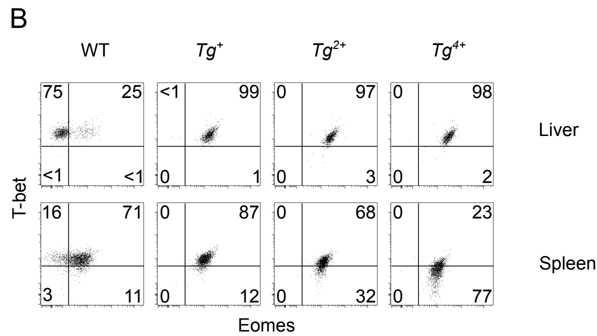


Figure 3.4. Eomes is repressive to T-bet expression by type 1 ILCs. (**A**) T-bet and Eomes expression by CD49a$^+$ DX5$^-$ (1), CD49a$^+$ DX5$^+$ (2), and CD49a$^-$ DX5$^+$ (3) type 1 ILC subsets from the livers and spleens of WT and *Tg*$^+$ mice (n=4 mice per genotype). (**B**) Flow cytometry of T-bet and Eomes expression by NK1.1$^+$ NKp46$^+$ type 1 ILCs from the livers and spleens of WT, *Tg*$^+$, *Tg*$^{2+}$, and *Tg*$^{4+}$ mice (n=3–4 mice per genotype). Data were obtained from mice that have 1 (*Tg*$^+$), 2 (*Tg*$^{2+}$), or 4 (*Tg*$^{4+}$) copies of the transgene as indicated. Data are mean ± SEM representative of 2–4 independent analyses; *p<0.05, **p<0.01, ***p<0.001.

effects of Eomes on T-bet expression appear to be dose-dependent because they are more apparent in transgenic lines with higher copy number and higher transgenic Eomes levels (Figure 3.4B).

Transgenic Eomes is permissive to some helper ILC1 attributes

Emerging evidence suggests that the progenitor cells of all helper ILCs are distinct from the progenitor cells of cNK cells. Helper ILC progenitors are marked by abundant expression of the transcription factor Id2, in contrast to cNK cell progenitors (Klose et al., 2014). We found that the descendant lineages of the distinct precursors, Eomes^{-} hILC1s versus Eomes^{+} cNK cells, continued to express high versus intermediate levels of Id2, respectively, across lymphoid and non-lymphoid organs (Figure 3.5A). We examined Id2 expression among type 1 ILCs using Id2 reporter mice in which human CD5 is expressed from an IRES-driven cassette knocked-in to the 3'-UTR of the *Id2* locus (Jones-Mason et al., 2012). Eomes^{-} hILC1s expressed higher levels of Id2 than Eomes^{+} cNK cells regardless of their maturation (Figure 3.5B). Like their adult counterparts, neonatal hILC1s also expressed higher levels of Id2 than neonatal cNK cells (Figure 3.5C). Neonatal cNK cells, however, appeared to have higher Id2 expression relative to adult cNK cells (Figure 3.5A, C), suggesting that the progenitor cells of cNK cells might have variable Id2 expression at different times during development.

Examination of transgenic NK1.1^{+} NKp46^{+} type 1 ILCs revealed a subpopulation of Id2^{hi} Eomes^{+} cells, suggesting the possibility that some cells arose from helper ILC precursors and were subsequently diverted into a cNK cell-like fate (Figure 3.5D). In support of the notion that the Id2^{hi} subpopulation may have arisen from helper ILC precursors, we detected other

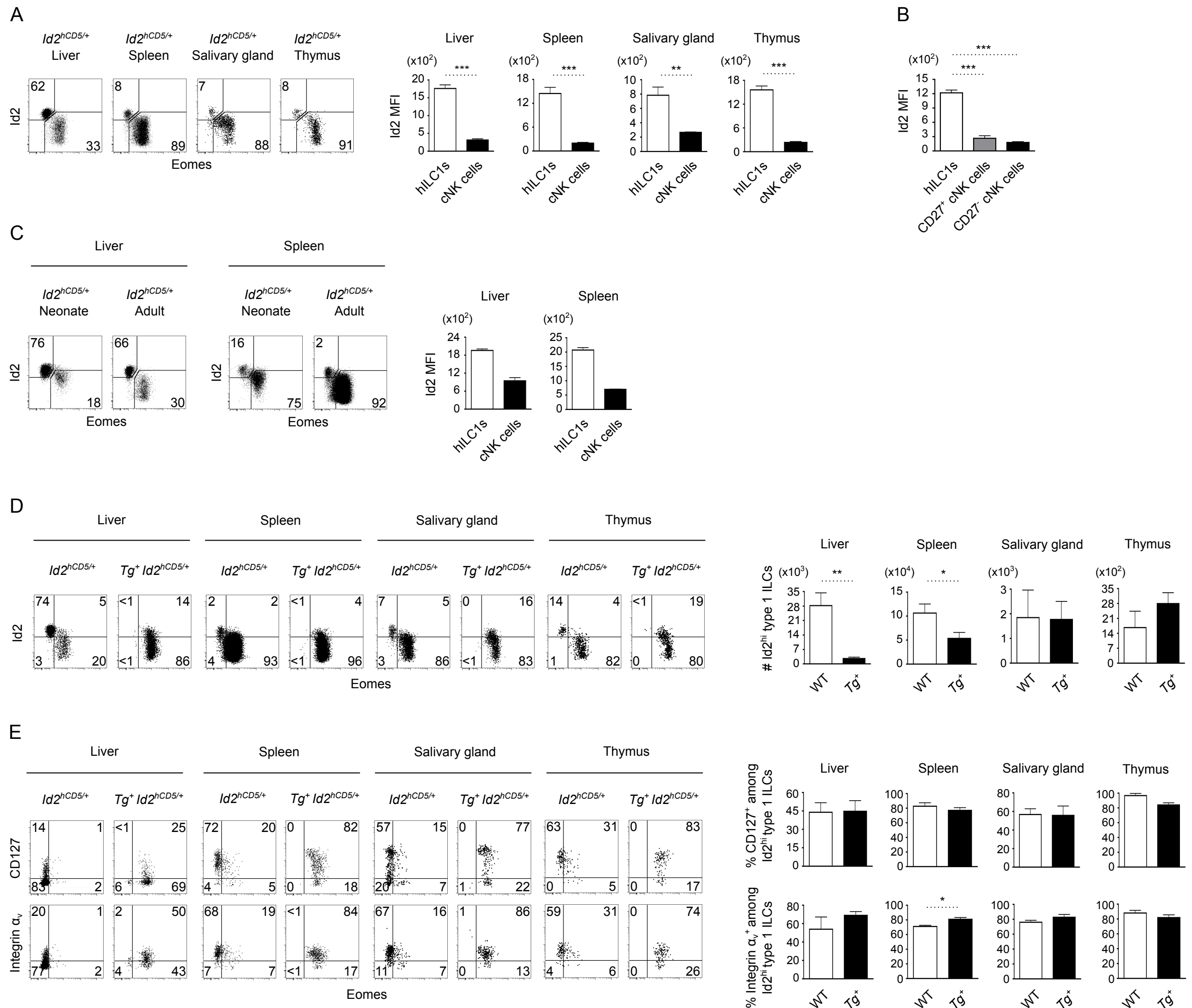


Figure 3.5. Eomes is permissive to some helper ILC1 attributes. (**A**) Flow cytometry of Id2 and Eomes expression by NK1.1$^+$ NKp46$^+$ type 1 ILCs from the indicated organs of $Id2^{hCD5/+}$ reporter mice (n=4 mice). Mean fluorescence intensity (MFI) of Id2 in CD49a$^+$ DX5$^-$ hILC1s and CD49a$^-$ DX5$^+$ cNK cells is indicated. (**B**) Expression of Id2 by CD49a$^+$ DX5$^-$ hILC1s, CD27$^+$ Eomes$^+$ immature cNK cells, and CD27$^-$ Eomes$^+$ mature cNK cells from the spleens of $Id2^{hCD5/+}$ mice (n=4 mice). (**C**) Flow cytometry of Id2 and Eomes expression by hepatic NK1.1$^+$ NKp46$^+$ type 1 ILCs from an $Id2^{hCD5/+}$ neonate (n=1 mouse) relative to an $Id2^{hCD5/+}$ adult. MFI of Id2 in neonatal hILC1s and cNK cells is shown. Results were validated in $Id2^{hCD5/hCD5}$ neonates bred to homozygosity for the *Id2* reporter allele (n=2 mice). (**D**) Flow cytometry of Id2 and Eomes expression by NK1.1$^+$ NKp46$^+$ type 1 ILCs from the indicated organs of $Id2^{hCD5/+}$ and Tg^+ $Id2^{hCD5/+}$ mice (n=4–5 mice per genotype). The number of Id2^{hi} cells is indicated. (**E**) Flow cytometry of CD127, integrin α_v, and Eomes expression by Id2^{hi} NK1.1$^+$ NKp46$^+$ type 1 ILCs from the indicated organs of $Id2^{hCD5/+}$ and Tg^+ $Id2^{hCD5/+}$ mice (n=3–4 mice per genotype). The frequency of Id2^{hi} cells that express CD127 and integrin α_v is indicated. Data are mean ± SEM representative of 3–5 independent analyses; *p<0.05, **p<0.01, ***p<0.001.

characteristics of hILC1s (Gordon et al., 2012; Klose et al., 2014; Takeda et al., 2005), including expression of CD127, integrin α_v, NKG2A/C/E receptors, and a restricted Ly49 receptor repertoire (Figure 3.5E, 3.6A). The Id2^{hi} subset of transgenic type 1 ILCs, nonetheless, also acquired numerous cNK cell features. Transgenic Eomes promoted upregulation of CD11b, CD43, KLRG1, and CD122 expression among Id2^{hi} cells in the liver, and to a lesser degree in the spleen (Figure 3.6B, C). Despite increased maturity, Id2^{hi} transgenic type 1 ILCs did not transition to the most terminal stages of cNK cell maturation (Figure 3.6B) marked by repression of CD27 (Chiossone et al., 2009). Together, these findings suggest that Eomes is sufficient to convert some but not all attributes of hILC1s to those of cNK cells.

Transgenic Eomes is permissive to helper features independently of T-bet

The persistence of some hILC1-like characteristics in a subset of transgenic type 1 ILCs could be the result of inadequate amounts of transgenic Eomes to compete against the unique functions of T-bet. To determine whether the helper features of the transgenic line are dependent on T-bet, we examined transgenic mice with genetic deletion of both endogenous *Tbx21* alleles. In the absence of an *Id2* reporter allele (Jones-Mason et al., 2012), we used Ly49^{-}NK1.1^{+} NKp46^{+} as surrogate markers for hILC1s. Transgenic Eomes was sufficient to partially rescue the development of cells with hILC1 features (expression of CD127 and integrin α_v) in *Tbx21*$^{-/-}$ mice (Figure 3.7A), indicating that the residual hILC1-like characteristics of the transgenic line are not likely to be T-bet-dependent. A similar subset with helper features was detected in the tissues of mice from transgenic lines with higher copy number and higher transgenic Eomes levels (Figure 3.7B). Furthermore, transgenic Eomes did not mobilize putative descendants of the hILC1 lineage to the blood, suggesting that helper-like cells in transgenic mice probably remain tissue-resident

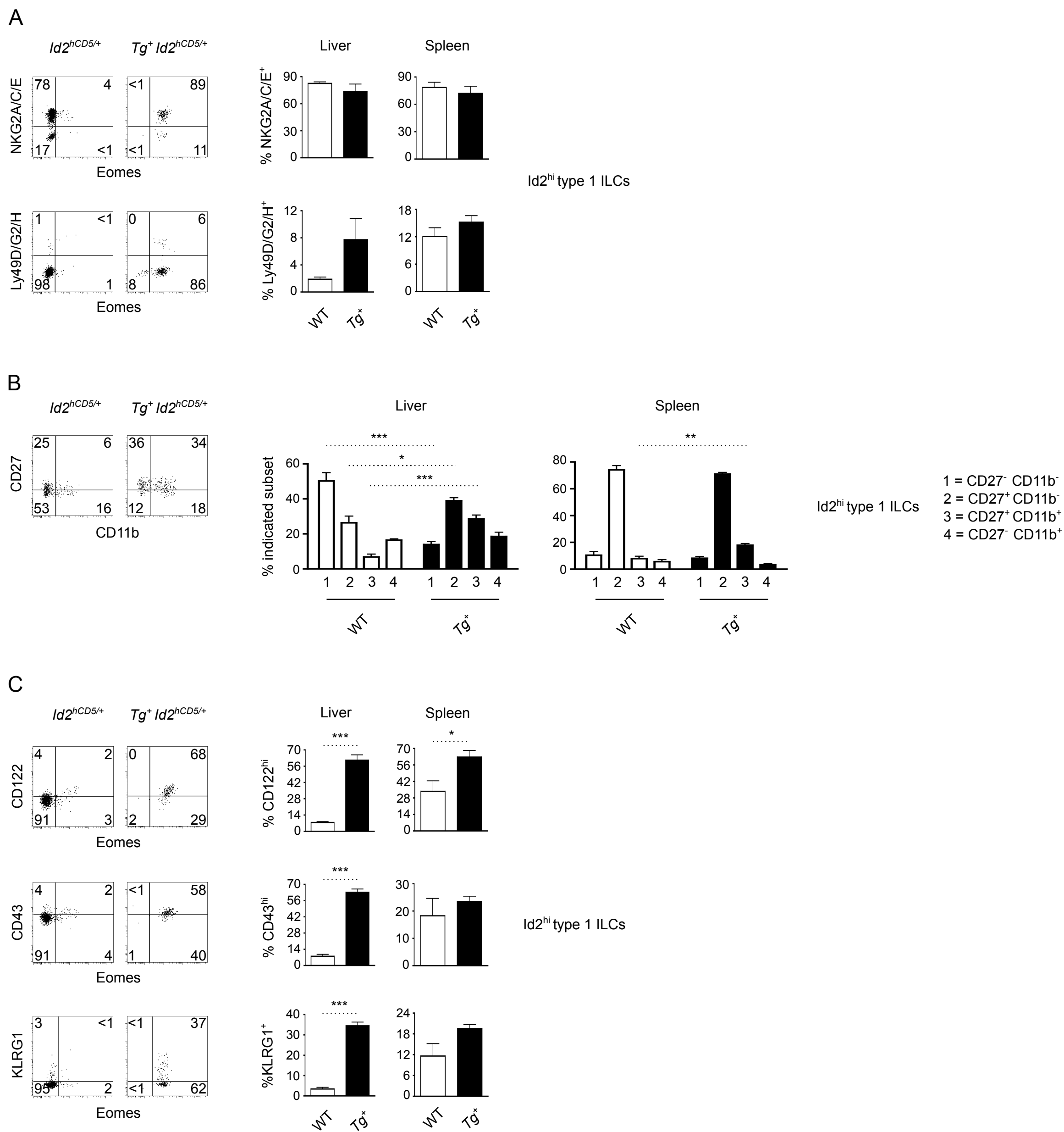


Figure 3.6. Eomes diverts putative descendants of the helper ILC1 lineage into a cNK cell-like fate. (**A**) Flow cytometry of NKG2A/C/E, Ly49D/G2/H, and Eomes expression by Id2^{hi} NK1.1^{+} NKp46^{+} type 1 ILCs from the livers of *Id2$^{hCD5/+}$* and *Tg^{+} Id2$^{hCD5/+}$* mice. Frequency of Id2^{hi} cells that express NKG2A/C/E and Ly49D/G2/H receptors in the liver and spleen is indicated. (**B**) Flow cytometry of CD27 and CD11b expression by Id2^{hi} type 1 ILCs from the livers of *Id2$^{hCD5/+}$* and *Tg^{+}Id2$^{hCD5/+}$* mice. Frequency of Id2^{hi} cells that progress through 4 stages of cNK cell maturation in the liver and spleen is shown. Stages defined by CD27 and CD11b expression are indicated. (**C**) Flow cytometry of CD122, CD43, KLRG1, and Eomes expression by Id2^{hi} type 1 ILCs from the livers of *Id2$^{hCD5/+}$* and *Tg^{+} Id2$^{hCD5/+}$* mice. Frequency of Id2^{hi} cells that express high levels of CD122, CD43, and KLRG1 in the liver and spleen is indicated. Data are mean ± SEM representative of 2 independent analyses with n=4 mice per genotype; *p<0.05, **p<0.01, ***p<0.001.

(Figure 3.7C). Our analyses of higher copy number transgenic lines support the notion that persistent hILC1-like features are not T-bet-dependent and do not result from inadequate amounts of transgenic Eomes to compete against T-bet. The ability of transgenic Eomes to partially compensate for T-bet in hILC1 development is somewhat analogous to the observation that residual hILC1s in *Tbx21*$^{-/-}$ mice and remnant cNK cells in *Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ mice are eliminated in mice with compound deficiency of T-bet and Eomes (see Chapter 2, Figure 2.4). The structural homology between Eomes and T-bet likely enables each factor to partially substitute for the other's function in development.

Transgenic T-bet does not induce hILC1 properties among type 1 ILCs

Our data suggest that Eomes is sufficient to divert type 1 ILC development into the cNK cell lineage, albeit incompletely, subsequent to divergent lineage specification of hILC1s and cNK cells from separate progenitors. To complement these findings, we also sought to determine whether enforced T-bet expression might be sufficient to redirect type 1 ILC development into the hILC1 lineage. We generated another novel transgenic mouse model in which T-bet codons are expressed according to *Tbx21* locus control elements, which is analogous to the strategy employed in the design of the Eomes transgenic mouse construct. We first verified that T-bet is expressed at higher than normal levels in the T-bet transgenic (*T-bet Tg*$^{+}$) mouse model by examining NK1.1^{+} NKp46^{+} populations where T-bet is ordinarily expressed (in the liver and spleen) or repressed (in the bone marrow). NK1.1^{+} NKp46^{+} type 1 ILCs had increased T-bet expression and reflexive Eomes repression to varying degrees across all tissues examined (Figure 3.8A), which is consistent with the previous suggestion that T-bet acts as a negative regulator of Eomes expression (Daussy et al., 2014; Gordon et al., 2012).

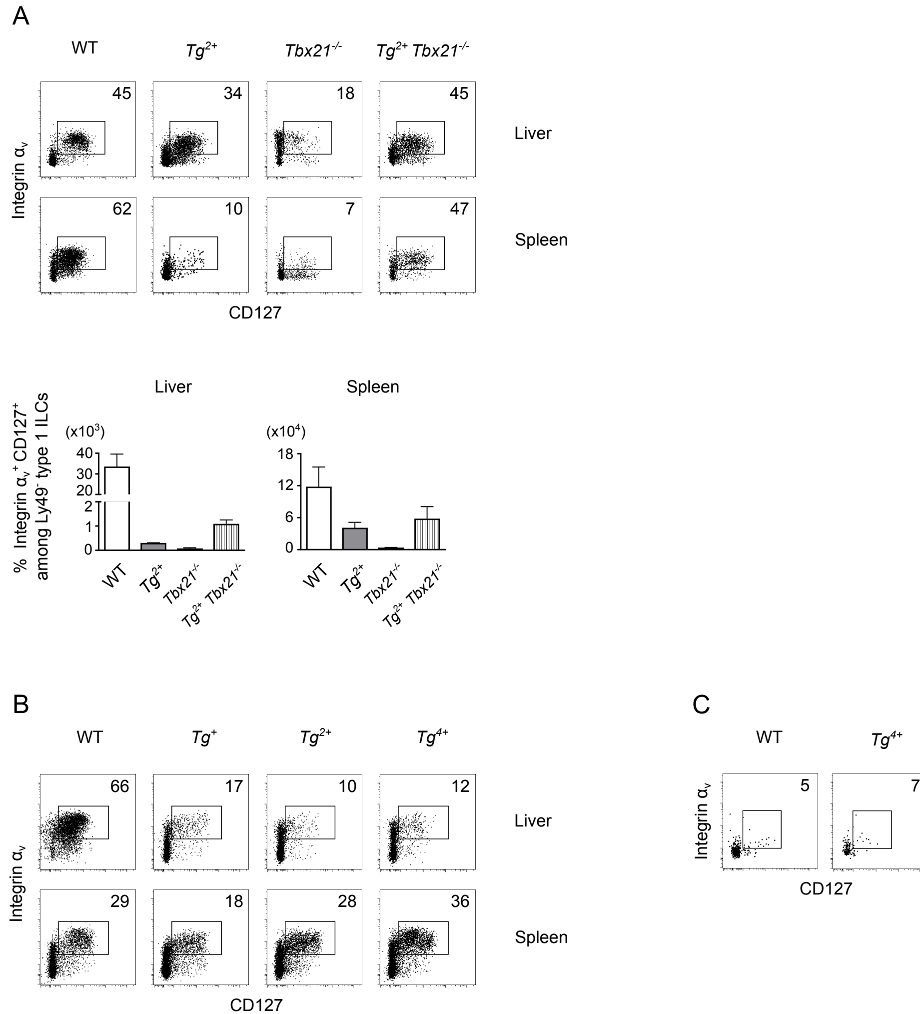


Figure 3.7. Eomes supports development of helper-like cells independently of T-bet. (**A**) Flow cytometry of CD127 and integrin α_v expression by Ly49$^-$ NK1.1$^+$ NKp46$^+$ type 1 ILCs from the livers and spleens of WT, *Tg*$^{2+}$, *Tbx21*$^{-/-}$, and *Tg*$^{2+}$ *Tbx21*$^{-/-}$ mice. Absolute numbers of hILC1-like (CD127$^+$ integrin $\alpha_v{}^+$) cells in the livers and spleens of n=2 mice per genotype are summarized. (**B**) Flow cytometry of CD127 and integrin α_v expression by Ly49$^-$ NK1.1$^+$ NKp46$^+$ type 1 ILCs from the livers and spleens of WT, *Tg*$^+$, *Tg*$^{2+}$, and *Tg*$^{4+}$ mice (n=2 mice per genotype). (**C**) Flow cytometry of CD127 and integrin α_v expression by Ly49$^-$ NK1.1$^+$ NKp46$^+$ type 1 ILCs from the blood of WT and *Tg*$^{4+}$ mice (n=2 mice per genotype). (A-C) Data were obtained from mice that have 1 (*Tg*$^+$), 2 (*Tg*$^{2+}$), or 4 (*Tg*$^{4+}$) copies of the transgene as indicated. Data are mean ± SEM representative of 2 independent analyses; *p<0.05, **p<0.01, ***p<0.001.

Contrary to our predictions, transgenic T-bet disrupted overall type 1 ILC development, in part due to impairment of hILC1 development (Figure 3.8B). Consistent with the inability of transgenic T-bet to promote CD49a^{+} DX5^{-} hILC1 development, we did not detect substantial upregulation of other hILC1 markers, such as Id2, CD127, and integrin α_v, despite evidence of Eomes repression (Figure 3.8C). We investigated the possibility that hILC1 development might be blocked at progenitor stages in T-bet transgenic mice. Enumeration of hILC1 progenitors and cNK cell progenitors (pre-NKPs and rNKPs) did not reveal an increased representation of transgenic progenitor populations, suggesting that transgenic T-bet likely does not block type 1 ILC differentiation at the progenitor stages (Figure 3.8D). Based on these findings, we speculate that transgenic T-bet might undermine hILC1 differentiation by impairing the survival of hILC1s. Transgenic T-bet also appears to be detrimental to cNK cell development by repressing Eomes expression during type 1 ILC maturation (Figure 3.8A). Therefore, transgenic T-bet seems to contribute to the overall decline of NK1.1^{+} NKp46^{+} type 1 ILCs and is not sufficient to redirect type 1 ILC development into the hILC1 lineage. Our results disagree with findings from a previous study, which demonstrated that transgenic T-bet expression driven by CD2 locus control elements was sufficient to promote development of Eomes^{-} cells at the expense of Eomes^{+} cells (Daussy et al., 2014). Differences in the aforementioned findings might be reconciled by the possibility that CD2 is expressed in hematopoietic progenitors (Siegemund et al., 2015), suggesting that CD2 might precede T-bet expression during development (Male et al., 2014). Ectopic T-bet expression in early hematopoietic progenitors could possibly have a different developmental outcome than enforced T-bet expression in developing type 1 ILCs following lineage specification.

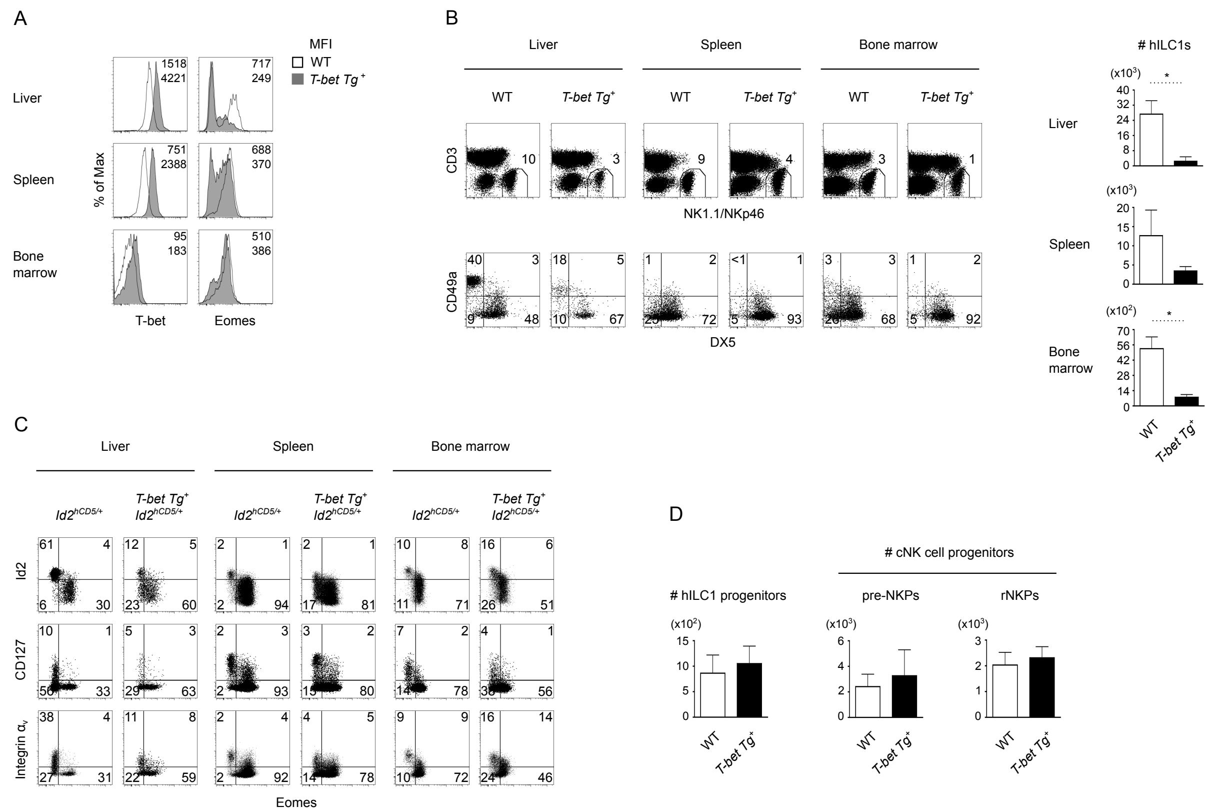


Figure 3.8. T-bet is not sufficient to induce helper ILC1 properties among type 1 ILCs. (**A**) T-bet and Eomes expression by NK1.1$^+$ NKp46$^+$ type 1 ILCs from the indicated organs of WT and *T-bet Tg*$^+$ mice (n=1 mouse per genotype). Results were validated in higher copy number transgenic lines (n=3 mice per genotype). (**B**) Flow cytometry of total type 1 ILCs, in addition to CD49a$^+$ DX5$^-$ hILC1 and CD49a$^-$ DX5$^+$ cNK cell subsets, from the indicated organs of WT and *T-bet Tg*$^+$ mice (n=3 mice per genotype). Absolute numbers of hILC1s are indicated. (**C**) Flow cytometry of Id2, CD127, integrin α_v, and Eomes expression by type 1 ILCs from the indicated organs of *Id2*$^{hCD5/+}$ and *T-bet Tg*$^+$ *Id2*$^{hCD5/+}$ mice (n=2 mice per genotype). (**D**) Absolute numbers of hILC1 progenitors [Lin (CD3, Gr-1, TER-119, CD19, NK1.1)$^-$ Id2$^+$ CD127$^+$ CD244$^+$ $\alpha_4\beta_7$$^+$ CD122$^-$ CD135$^-$] and cNK cell progenitors [pre-NKPs = Lin$^-$ Id2$^-$ CD127$^+$ CD244$^+$ CD122$^-$ CD135$^-$ and rNKPs = Lin$^-$ Id2$^-$ CD127$^+$ CD244$^+$ CD122$^+$ CD135$^-$] from the bone marrow of *Id2*$^{hCD5/+}$ and *T-bet Tg*$^+$ *Id2*$^{hCD5/+}$ mice (n=3 mice per genotype). Data are mean ± SEM representative of 2–3 independent analyses; *p<0.05, **p<0.01, ***p<0.001.

This study suggests that type 1 ILCs with distinct lineage origins have the potential for substantial developmental plasticity. Our data is consistent with a model in which separate type 1 ILC lineages are specified from distinct precursors and the absence or presence of Eomes subsequently directs or switches, albeit incompletely, their determination as hILC1s or cNK cells. In an analogous manner, $CD4^+$ T cells can acquire cytotoxic T cell attributes and lose helper attributes when ThPOK expression decreases and Runx3 expression increases, yet they can still continue to express the CD4 marker (Mucida et al., 2013; Reis et al., 2013). Conversely, $CD8^+$ T cells can acquire some helper T cell features and lose expression of cytotoxic features when cKrox expression increases (Jenkinson et al., 2007). Understanding the nature of the inter-relatedness and plasticity between type 1 ILC subsets will help harness their therapeutic potential in infection, inflammation, and cancer. In this study, genetic manipulation of type 1 ILC subsets resulted in the derivation of novel helper-like cells with heritable Ly49 receptor expression as well as cNK-like cells with remnant helper attributes. The hybrid nature of these novel populations could help accelerate progress towards the clinical use of type 1 ILC-based therapy.

MATERIALS AND METHODS

Mice

Mice were housed in specific pathogen-free conditions and used in accordance with the Columbia University Institutional Animal Care and Use Committee's guidelines. C57BL/6 *Tbx21*$^{-/-}$ (Intlekofer et al., 2005) and *Id2*$^{hCD5/hCD5}$ reporter (Jones-Mason et al., 2012) mice were generated as previously described. Generation of novel transgenic mouse lines is described below. Neonatal mice (0-14 days of age) and adult mice (4-16 weeks of age) were used for analyses.

Detailed characterization of the *Tbx21-Eomes* transgenic and *Tbx21-Tbx21-eGFP* transgenic mouse strains will appear elsewhere. Unlike the *Tbx21-Eomes* or Eomes transgenic mouse strain, the *Tbx21-Tbx21-eGFP* or T-bet transgenic mouse strain is a T-bet-GFP fusion reporter. Briefly, to generate the *Tbx21-Eomes* or Eomes transgenic mouse strain, Eomes cDNA followed by an SV40-PolyA site was inserted into the ATG translational start site of T-bet in the *Tbx21* BAC clone RP23-237M14 (CHORI) by Red/ET recombination technology (Gene Bridges). This strategy enabled T-bet regulatory elements to drive Eomes expression instead of T-bet expression from the transgenic allele. The BAC construct was used for pronuclear injection of C57BL/6 zygotes. To generate the T-bet transgenic mouse strain, Tbx21 followed by eGFP cDNA was inserted into the ATG translational start site of T-bet in the *Tbx21* BAC clone RP23-237M14 (CHORI) by Red/ET recombination technology (Gene Bridges). The BAC construct was used for pronuclear injection of B6CBA/Fl zygotes and transgenic mice were subsequently backcrossed to C57BL/6 mice for multiple generations. Experimental phenotypes in both Eomes and T-bet transgenic mice were identified using lines that carried 1 copy of the transgene and

verified in higher copy number lines that carried 2 or 4 copies of the transgene. For some experiments, transgenic mice were crossed to *Id2*$^{hCD5/hCD5}$ reporter mice that express human CD5 from an IRES-driven cassette knocked-in to the 3'-UTR of *Id2* (Jones-Mason et al., 2012). Furthermore, Eomes transgenic mice were crossed to *Tbx21*$^{-/-}$ mice for some analyses. Aged transgenic mice had no evidence of skin lesions, intestinal inflammation, or hepatosplenomegaly.

Lymphocyte isolation and flow cytometry

Livers were dissected and thoroughly perfused with PBS. Single-cell suspensions were prepared from freshly harvested liver, spleen, and thymus after mechanical dissociation and passage through 70 μm cell strainers. Livers were subsequently resuspended in 40% Percoll at room temperature (GE Healthcare) and centrifuged over 60% Percoll at 2000 rpm for 20 minutes. Liver lymphocytes were isolated at the interphase of the Percoll gradient. Red blood cells in spleen and thymus suspensions were lysed using ACK lysing buffer (ThermoFisher Scientific).

Freshly harvested salivary glands were cut into small pieces and digested in the presence of DNase I and either collagenase D or liberase TL (Roche Life Science) for 1 hour, as suggested in previous studies (Cortez et al., 2014; Sojka et al., 2014). Following digestion, single-cell suspensions were prepared after mechanical dissociation and passage through 70 μm cell strainers. Red blood cells in salivary gland suspensions were lysed using ACK lysing buffer (ThermoFisher Scientific).

Cells were stained with a LIVE/DEAD fixable dead cell stain kit (Invitrogen) and blocked with antibodies against CD16/CD32 (BD) prior to staining with cell surface antibodies for CD3,

CD11b, CD27, CD43, CD122, human CD5, integrin α_v, KLRG1, Ly49G2, Ly49H, NKp46, and TRAIL (eBioscience); CD19, CD49a, CD49b, CD127, Ly49D, Ly-6G/Ly-6C (Gr-1), NK1.1, NKG2A/C/E, and TER-119 (BD). Cells were treated with eBioscience or BD cytofix/cytoperm kits prior to staining with antibodies for the transcription factors T-bet (eBioscience) and Eomes (eBioscience) or the cytokine TNF-α (BD). Cells were analyzed on an LSRFortessa coupled to FACSDiva software. Data were analyzed with FlowJo software (Treestar).

Adoptive transfer studies

Hepatic CD45.1 lymphocytes were depleted of T cell, B cell, granulocyte, and erythroid cell lineages using an NK cell isolation kit (Miltenyi Biotec) and MACS manual magnet separation. Following NK cell enrichment, cells were stained with antibodies for CD3, NK1.1, NKp46, CD49a, CD49b, Ly49A, Ly49D, Ly49G2, and Ly49H. 5×10^5 – 1×10^6 CD49a$^+$ DX5$^-$ Ly49$^-$ hILC1s were sorted on a FACSAriaII (BD) to a purity of at least 95%. Purified CD45.1 hILC1s were transferred intravenously into unirradiated CD45.2 *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ recipients for 24 hours.

Temporal deletion studies

Splenic lymphocytes were depleted of T cell, B cell, granulocyte, and erythroid cell lineages using an NK cell isolation kit (Miltenyi Biotec) and MACS manual magnet separation. Following NK cell enrichment, cells were stained with antibodies for NK1.1, NKp46, and CD49b. 1×10^6 NK1.1$^+$ NKp46$^+$ DX5$^+$ cNK cells were sorted on a FACSAriaII to a purity of at least 95%. Purified cNK cells were washed thoroughly in serum-free media and subdivided into separate groups. One group was treated with media alone (sham) while the other group was treated with 50 μg/mL Cre recombinase fused to the HIV TAT protein transduction domain

(Wadia et al., 2004), also known as TAT-Cre (obtained from The Protein and Proteomics Core Facility of The Children's Hospital of Philadelphia). Reactions were incubated for 45 minutes at 37°C and subsequently quenched by addition of media containing 10% FCS and thorough washing. TAT-Cre or sham-treated cells were injected intravenously into separate unirradiated *Il2rg*$^{-/-}$ *Rag2*$^{-/-}$ recipients for 7-10 days.

For in vitro temporal deletion studies, 1 x 10^6 highly purified splenic cNK cells were treated with TAT-Cre reaction or media prior to being cultured in RPMI 1640 supplemented with 10% FCS and 2,000 U/mL rIL-2. On day 5, cells were washed and stained with antibodies for NK1.1, NKp46, TRAIL, and CD49b. $TRAIL^+$ $DX5^-$ helper-like cells were sorted according to expression of Ly49D, Ly49H, or both. The 3 cell populations were placed in culture with 2,000 U/mL rIL-2 prior to analysis on day 10.

In vitro stimulation

To analyze T-bet and Eomes expression by $CD4^+$ T cells, splenocytes from WT and *Tg*$^+$ mice were stimulated at a concentration of 3 x 10^6 cells/mL in 24 well plates with anti-CD3 and anti-CD28 for 48 hours. To analyze TNF-α production, 5 x 10^5 – 1 x 10^6 hepatic lymphocytes from WT and *Tg*$^+$ mice were stimulated in round-bottomed 96 well plates with 200 ng/mL PMA and 5 μg/mL ionomycin in the presence of GolgiPlug (Invitrogen) for 4 hours.

Statistical analysis

Statistics were calculated using Prism (GraphPad Software). Differences between groups were quantified using the unpaired *t*-test. Statistical significance was reached at $p < 0.05$.

CHAPTER 4

DISCUSSION

Summary

In this thesis work, I found that the transcription factor Eomes plays its essential role in cNK cell maturation after the onset of NKp46 expression. Eomes and its homolog T-bet are required in order to develop the spectrum of lymphoid and non-lymphoid type 1 ILCs. My findings revealed, furthermore, that type 1 ILCs with distinct lineage origins have the potential for substantial developmental plasticity. Temporal deletion of Eomes directed cNK cells toward an hILC1-like fate in a T-bet-dependent manner. Ectopic Eomes expression according to *Tbx21* locus control redirected hILC1s into a cNK cell-like fate independently of T-bet. Enforced T-bet expression according to *Tbx21* locus control, however, was not sufficient to direct type 1 ILC development into the hILC1 lineage. These data are consistent with a model in which Eomes acts as a key determinant of cNK cell versus hILC1 maturation following lineage specification of cNK cells and hILC1s from distinct precursors. Subsequent studies will determine how early ILC specification factors (Figure 4.1) might be linked to the induction of T-bet and Eomes expression in developing type 1 ILCs. Future identification of the differential gene targets of T-bet and Eomes will also enhance our understanding of how homologous T-box factors perform unique functions during type 1 ILC development.

Refined hematopoietic model for ILC development

All ILCs develop from common lymphoid progenitors (CLPs) in multiple stages during which ILC lineages are specified and alternative lymphocyte fates are suppressed. A complex hierarchy of transcription factors orchestrates early ILC fate specification and ultimately regulates

commitment to specific ILC lineages. The transcriptional network that underlies ILC development is currently being elucidated. The transcription factors NFIL3 and TOX, which are expressed by all mature ILC lineages, have emerged as central regulators of early ILC lineage specification. NFIL3 and TOX regulate expression of Id2 (Aliahmad et al., 2010; Male et al., 2014; Seehus et al., 2015; Xu et al., 2015), which is critical for the development of all ILC lineages (Boos et al., 2007; Eberl et al., 2004; Monticelli et al., 2011; Moro et al., 2010; Vosshenrich and Di Santo, 2013; Yokota et al., 1999). NFIL3 directly activates Id2 expression in common helper innate lymphoid progenitors (CHILPs), which is consistent with the ability of Id2 to rescue the development of all ILC lineages from *Nfil3*$^{-/-}$ hematopoietic progenitors (Gascoyne et al., 2009; Male et al., 2014; Xu et al., 2015). Id2 also rescues PLZF expression in *Nfil3*$^{-/-}$ CHILPs (Xu et al., 2015) and PLZF is critical for development of most helper ILCs (Constantinides et al., 2014). Expression of NFIL3, Id2, and PLZF, therefore, appear to be linked. Whether Id2 can rescue the development of helper ILC lineages from *Tox*$^{-/-}$ progenitors has yet to be determined. It has previously been suggested that Id2 expression in *Tox*$^{-/-}$ progenitors fails to rescue cNK cell development (Aliahmad et al., 2010), perhaps because cNK cell lineage specification occurs independently of Id2 (Boos et al., 2007; Klose et al., 2014).

NFIL3 might be genetically upstream of TOX induction because CLPs express NFIL3, but appear to lack substantial TOX expression (Male et al., 2014; Seehus et al., 2015; Yu et al., 2014). TOX expression is downregulated in *Nfil3*$^{-/-}$ CLPs while NFIL3 expression is unchanged in *Tox*$^{-/-}$ ILC progenitors (Seehus et al., 2015; Yu et al., 2014), which supports the notion that NFIL3 regulates TOX. There is also evidence to suggest that TOX expression in *Nfil3*$^{-/-}$ hematopoietic progenitors rescues the development of all ILC lineages (Yu et al., 2014).

Furthermore, NFIL3 appears to directly activate TOX expression in a mouse lymphoma cell line (Yu et al., 2014). The relationship between TOX and NFIL3, however, might be more complex. If NFIL3 acts upstream of TOX during ILC development, it is not yet understood why lymphoid tissue organogenesis is abrogated in *Tox*$^{-/-}$ but not in *Nfil3*$^{-/-}$ mice (Aliahmad et al., 2010; Seillet et al., 2014b). Future studies will continue to elucidate the regulatory network involving NFIL3, TOX, and Id2.

In light of the recent identification of progenitors to helper ILC lineages (CHILPs) (Constantinides et al., 2014; Klose et al., 2014), there has been considerable interest in characterizing upstream common innate lymphoid progenitors (CILPs) to all ILC lineages. The precise identity of CILPs remains elusive, but will be critical for our understanding of how hILC1 and cNK cell lineages branch during development. NFIL3 activity in CLPs has been suggested to promote emergence of integrin $\alpha_4\beta_7^+$ progenitors to all ILCs, which are marked by expression of the chemokine receptor CXCR6 (Possot et al., 2011; Yu et al., 2014). Similar to CHILPs, CXCR6$^+$ progenitors lack expression of ILC lineage-defining factors, such as T-bet, GATA3, RORγt, and Eomes. CXCR6$^+$ progenitors appear to be less differentiated than CHILPs because they have low levels of Id2 and negligible PLZF expression (Yu et al., 2014). Moreover, it is possible that TOX might be genetically upstream of CXCR6 induction in CILPs. TOX deficiency impairs the generation of CHILPs and results in developmental arrest at a transitional state that presumably occurs between the CLP and CHILP stages (Seehus et al., 2015). Remnant *Tox*$^{-/-}$ progenitors resemble putative CILPs that express low levels of Id2 and CD127. Significant reduction in CXCR6 expression among *Tox*$^{-/-}$ progenitors is consistent with the possibility that TOX activates CXCR6 expression in putative CILPs (Seehus et al., 2015). Future studies that

examine the precursor-product relationships between CLPs, CXCR6$^+$ progenitors, and CHILPs will be critical to understanding the steps required for continued ILC lineage specification.

Because only 2.5% of CXCR6$^+$ pan-ILC precursors differentiate into all ILC lineages (Yu et al., 2014), another putative CILP has recently been proposed (Yang et al., 2015). The majority of pan-ILC progenitors marked by TCF-1 expression efficiently give rise to all helper ILC subsets and cNK cells. TCF-1$^+$ progenitors express low levels of Id2 and CD127, but lack substantial CXCR6 expression like the other precursors proposed as putative CILPs (Yang et al., 2015; Yu et al., 2014). TCF-1$^+$ progenitors closely resemble CLPs at the transcriptome level, but appear to be more differentiated because they cannot give rise to B cells and T cells (Yang et al., 2015). TCF-1$^+$ progenitors have differentiation potential for dendritic cells in addition to ILCs, which suggests that they are not purely ILC precursors (Yang et al., 2015). Development of TCF-1$^+$ progenitors persists in *Id2*$^{-/-}$ mice, while development of CHILPs is diminished in *Id2*$^{-/-}$ mice and *Tcf7*$^{-/-}$ (TCF-1-deficient) mice (Yang et al., 2015). Therefore, TCF-1$^+$ progenitors appear to precede CHILPs during ILC development, which is consistent with the possibility that TCF-1 could be genetically upstream of Id2. The relationship between TCF-1 and Id2 is complicated by the fact that TCF-1$^+$ progenitors appear to develop less efficiently in the absence of Id2 (Kaye, 2015; Yang et al., 2015). Further investigation of the regulatory network involving TCF-1 and Id2 is warranted. Future studies are also needed to closely compare TCF-1$^+$ and CXCR6$^+$ pan-ILC progenitors and determine their precursor-product relationship.

The transcription factor Ets-1, which regulates cNK cell lineage specification and development (Barton et al., 1998; Ramirez et al., 2012), has recently been implicated in helper ILC

differentiation (Eberl et al., 2015). Because Ets-1 binds to the *Id2* locus in NKPs and cNK cells (Ramirez et al., 2012), there might be a role for Ets-1 in the emergence of pan-ILC progenitors (CILPs) that have low Id2 expression and CHILPs that have abundant Id2 expression (Klose et al., 2014; Xu et al., 2015; Yu et al., 2014). Ets-1 appears to be downstream of NFIL3 based on its ability to rescue type 1 ILC development from *Nfil3*$^{-/-}$ hematopoietic progenitors (Male et al., 2014). It has yet to be determined whether PU.1, another member of the ETS family of transcription factors, plays a role in the development of helper ILC lineages. Similar to Ets-1, PU.1 is required for the lineage specification and development of cNK cells, but PU.1 does not appear to regulate Id2 and TCF-1 expression in cNK cells (Colucci et al., 2001). If PU.1 is part of the network of factors that regulate early ILC lineage specification, PU.1 might function downstream of the TCF-1$^+$ CILP and Id2$^+$ CHILP stages.

Based on recent reports of transcription factors responsible for early ILC lineage specification, one might propose that the following stages precede commitment to specific ILC lineages (Figure 4.1). Notch signaling and NFIL3 expression in CLPs result in the development of $\alpha_4\beta_7^+$ progenitors that upregulate TOX and express low levels of Id2 (Seehus et al., 2015; Xu et al., 2015; Yu et al., 2014). Because *Tox*$^{-/-}$ ILC progenitors have reduced expression of genes that encode Notch family members, TOX appears to serve as a crucial link between Notch signaling and downstream target genes, such as TCF-1 (Seehus et al., 2015). TCF-1 expression marks $\alpha_4\beta_7^+$ CILPs that can efficiently give rise to all ILC lineages (Yang et al., 2015). TOX might also promote expression of CXCR6 in another $\alpha_4\beta_7^+$ pan-ILC precursor population (Seehus et al., 2015; Yu et al., 2014). In contrast to TCF-1$^+$ progenitors, CXCR6$^+$ progenitors are likely a heterogeneous population because a minority of them give rise to all ILC lineages (Yang et al.,

2015; Yu et al., 2014). TCF-1$^+$ CXCR6$^-$ and CXCR6$^+$ pan-ILC precursors appear to be distinct populations whose relationship remains unclear (Yang et al., 2015). Subsequent to their development, $\alpha_4\beta_7^+$ CILPs presumably downregulate expression of Notch family members because sustained Notch signaling might favor T cell at the expense of ILC lineage specification (Seehus et al., 2015). The transition from CILPs to CHILPs is, therefore, thought to occur independently of Notch signaling (Kaye, 2015; Seehus et al., 2015) under the control of NFIL3, TOX, and TCF-1 (Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015; Yu et al., 2014). CHILPs have abundant expression of all three factors, which is consistent with the notion that NFIL3, TOX, and TCF-1 regulate their emergence (Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015). NFIL3 expression in CD127-expressing CHILPs is controlled by the common γ-chain-dependent cytokine IL-7 (Xu et al., 2015). Furthermore, NFIL3 directly activates Id2 expression in CHILPs, while TOX achieves a similar effect either directly or indirectly (Seehus et al., 2015; Xu et al., 2015). Although NFIL3, TOX, and TCF-1 appear to be upstream of Id2, their relationship might be more complex than is currently appreciated because NFIL3 and TOX expression is decreased in *Id2*$^{-/-}$ TCF-1$^+$ progenitors (Yang et al., 2015).

The developmental divergence of Id2$^+$ progenitors of hILC1s (CHILPs) and Id2$^-$ progenitors of cNK cells (NKPs) occurs downstream of the putative CILP stage (Klose et al., 2014). Helper ILC lineages (hILC1s, hILC2s, hILC3s) arise from CHILPs that upregulate the transcription factor PLZF and lose the potential to differentiate into lymphoid tissue inducer (LTi) cells (Constantinides et al., 2014; Klose et al., 2014). GATA-3 is critical for the development of helper ILCs and dispensable for the differentiation of cNK cells (Yagi et al., 2014). Furthermore, GATA-3 does not appear to regulate Id2 expression (Yagi et al., 2014), suggesting that GATA-3

likely acts downstream of the bifurcation between CHILPs and NKPs to promote development of helper ILC lineages from CHILP descendants (Figure 4.1). Thus far, the generation of NKPs is thought to depend on NFIL3 and TCF-1 (Male et al., 2014; Seillet et al., 2014a; Yang et al., 2015). Although a previous study has suggested otherwise (Aliahmad et al., 2010), a potential role for TOX in NKP development should be revisited using the most current definition of NKPs (Carotta et al., 2011; Fathman et al., 2011). Alternatively, it is possible that TOX, like Id2, is not required for cNK cell lineage specification (Aliahmad et al., 2010; Boos et al., 2007).

Potential regulators of T-bet and Eomes during ILC development

It remains unclear how transcription factors expressed by early ILC progenitors might promote expression of ILC lineage-identifying factors. Factors that directly bind to the *Tbx21* locus in type 1 ILCs are mostly unknown and will be identified in future chromatin immunoprecipitation (ChIP) studies. As a notable exception, Ets-1 binds to the *Tbx21* locus in cNK cells (Ramirez et al., 2012), although its role in early ILC development has yet to be elucidated. In support of the notion that T-bet is a direct target of Ets-1, *Ets1*$^{-/-}$ NKPs had reduced expression of T-bet (Ramirez et al., 2012).

T-bet expression might be regulated by factors that perform similar functions to T-bet during type 1 ILC development (Daussy et al., 2014; Gordon et al., 2012; Klose et al., 2014; Sojka et al., 2014). GATA-3 is one candidate regulator of T-bet that is required for hILC1 development, but dispensable for cNK cell differentiation (Samson et al., 2003; Yagi et al., 2014). Because GATA-3 expression is detectable in early post-CLP stages (Constantinides et al., 2014; Tindemans et al., 2014), GATA-3 appears to precede T-bet expression during ILC development

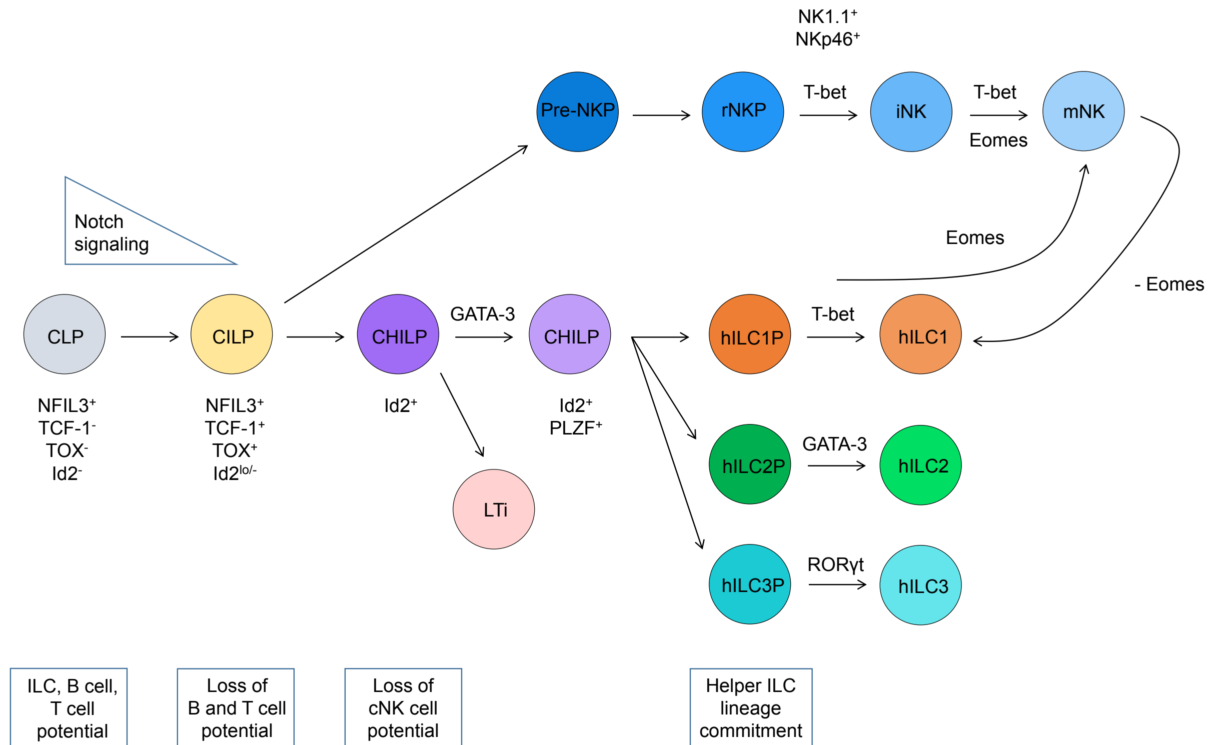


Figure 4.1. Refined hematopoietic map for the development of ILC lineages. Notch signaling and NFIL3 expression in CLPs result in emergence of $\alpha_4\beta_7^+$ common innate lymphoid progenitors (CILPs) that express TOX, TCF-1, and low levels of Id2 (Kaye, 2015; Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015; Yu et al., 2014). In contrast to CLPs, CILPs have the potential to differentiate into all ILC lineages, but not B cell and T cell lineages. CILPs downregulate expression of Notch receptors to potentially suppress an alternative T cell fate. Therefore, progression from CILPs to common helper innate lymphoid progenitors (CHILPs) is thought to occur independently of Notch signals (Kaye, 2015; Seehus et al., 2015). CHILPs develop under the control of NFIL3, TOX, and TCF-1. NFIL3 and TOX are genetically upstream of Id2 induction in CHILPs (Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015; Yu et al., 2014). Because CHILPs lose the potential to differentiate into cNK cells, progenitors of cNK cells (pre-NKPs) likely branch off upstream of CHILPs at the CILP stage (Klose et al., 2014). CHILPs that upregulate PLZF expression lose the potential to develop into lymphoid tissue inducer (LTi) cells and give rise to progenitor cells that are committed to the T-bet-dependent ILC1, GATA-3-dependent ILC2, and RORγt-dependent ILC3 helper lineages (Constantinides et al., 2014; Klose et al., 2014). After branching off upstream of CHILPs, pre-NKPs differentiate into refined NKPs (rNKPs), which give rise to cNK cells that progress through immature (iNK) and mature (mNK) stages of development under the control of T-bet and Eomes. Ectopic Eomes expression according to *Tbx21* locus control redirects cells specified from the hILC1 lineage toward a cNK cell-like identity. Temporal deletion of Eomes from cNK cells triggers their progression toward an hILC1-like fate.

(Gordon et al., 2012; Townsend et al., 2004). GATA-3 and T-bet are expressed by hILC1s and cNK cells (Gordon et al., 2012; Samson et al., 2003; Yagi et al., 2014), which is consistent with the possibility that GATA-3 might enable T-bet expression during the development of both type 1 ILC lineages. GATA-3-deficient cNK cells have reduced T-bet expression, suggesting that GATA-3 might be genetically upstream of T-bet induction (Samson et al., 2003). GATA-3 expression was not affected in T-bet-deficient cNK cells (Townsend et al., 2004). GATA-3 and T-bet cooperate to regulate maturation and function during type 1 ILC differentiation, but assume opposing roles during T helper cell differentiation (Di Santo, 2006; Samson et al., 2003; Townsend et al., 2004). T-bet and GATA-3 compete with each other to establish Th1 cell-specific versus Th2 cell-specific gene expression programs in naïve $CD4^{+}$ T cells upon activation (Lazarevic et al., 2013). There is no current evidence to suggest that GATA-3 directly binds to the *Tbx21* locus in T cells. However, T-bet has been shown to interact with GATA-3, resulting in the redistribution of GATA-3 away from Th2 cell-specific genes and toward T-bet-binding sites at Th1 cell-specific genes (Hwang et al., 2005; Kanhere et al., 2012). Protein-protein interactions between T-bet and GATA-3 in type 1 ILCs are another area of interest for future studies.

PLZF might serve as another putative regulator of T-bet expression. PLZF precedes T-bet expression during ILC development because PLZF is expressed in CHILP descendants (Constantinides et al., 2014; Gordon et al., 2012; Townsend et al., 2004). Based on analyses of *Zbtb16*$^{-/-}$ mice that lack PLZF protein, it was suggested that PLZF plays an analogous role to T-bet in hILC1 development, but appears to be dispensable for cNK cell differentiation (Constantinides et al., 2014). Consistent with data from *Zbtb16*$^{-/-}$ mice, PLZF lineage tracing experiments revealed that hILC1s are prominently marked with a history of PLZF expression,

while cNK cells are labeled to a lesser degree (Constantinides et al., 2014). Examination of remnant hILC1s in *Zbtb16*$^{-/-}$ mice revealed lower expression of CD127 (Constantinides et al., 2015), while expression of other hILC1-associated markers, such as CD49a, TRAIL, and CXCR6 (Paust et al., 2010; Peng et al., 2013; Takeda et al., 2005), was largely unaffected (Constantinides et al., 2015). Previous data from our lab suggests that T-bet is genetically upstream of CD127, CD49a, TRAIL, and CXCR6 induction. Genetic deletion of PLZF does not completely phenocopy T-bet deficiency, suggesting that the relationship between T-bet and PLZF might be complex and involve a network of other factors. Future analyses in *Zbtb16*$^{-/-}$ mice can help determine whether PLZF is linked to T-bet expression in type 1 ILCs.

Finally, Id2 might be an upstream regulator of T-bet expression. In contrast to GATA-3 and PLZF, Id2 is required for the generation of all ILC lineages (Boos et al., 2007; Eberl et al., 2004; Monticelli et al., 2011; Moro et al., 2010; Vosshenrich and Di Santo, 2013; Yokota et al., 1999). CHILPs are marked by abundant Id2 expression and their hILC1 progeny continue to express high levels of Id2 as they upregulate T-bet expression (Gordon et al., 2012; Klose et al., 2014; Townsend et al., 2004). In contrast, Id2 appears to be upregulated concurrently with T-bet in cNK cells subsequent to their lineage specification from NKPs (Gordon et al., 2012; Klose et al., 2014; Townsend et al., 2004). Comprehensive analyses of type 1 ILC development in *Id2*$^{-/-}$ mice (Boos et al., 2007), however, predate the characterization of CD49a-expressing hILC1s (Peng et al., 2013) and the identification of the earliest NK lineage-committed progenitors (NKPs) (Carotta et al., 2011; Fathman et al., 2011). Examination of remnant type 1 ILC subsets and progenitor populations in *Id2*$^{-/-}$ mice will be critical for uncovering putative targets of Id2.

Expressed in early ILC progenitors and mature type 1 ILCs, the transcription factors NFIL3, TOX, and TCF-1 might be upstream regulators of Eomes expression (Seehus et al., 2015; Xu et al., 2015; Yang et al., 2015). Using ChIP analysis in a murine cell line, a recent study revealed that NFIL3 directly binds to the *Eomes* locus (Male et al., 2014). *Nfil3*$^{-/-}$ cNK cells exhibited reduced Eomes expression, while T-bet expression did not appear to be affected. Eomes rescued type 1 ILC development from *Nfil3*$^{-/-}$ hematopoietic progenitors more efficiently than NFIL3 and other factors, which is consistent with the idea that NFIL3 is genetically upstream of Eomes induction (Male et al., 2014; Seillet et al., 2014a). NFIL3 is also expressed by Eomes^{-} hILC1s and has been implicated in hILC1 development (Xu et al., 2015; Yu et al., 2014). Therefore, it remains unclear how and why Eomes expression is enabled in cNK cells, but not in hILC1s. One study suggested that hILC1s develop independently of NFIL3 in the liver, in contrast to cNK cells (Seillet et al., 2014a). Type 1 ILC lineages might have differential requirements for NFIL3 in some organs, which could help explain why Eomes expression is confined to cNK cells. It has also been reported that salivary gland type 1 ILCs express Eomes independently of NFIL3 (Cortez et al., 2014), suggesting that alternative factors regulate Eomes expression. It has yet to be determined whether TCF-1 is linked to Eomes expression. However, a recent study has suggested that *Tox*$^{-/-}$ progenitors had a substantial reduction in Eomes expression, while T-bet expression appeared to be unchanged (Seehus et al., 2015).

In agreement with previous reports, this study suggests that Eomes cannot be rapidly de-repressed in hILC1s displaced from their tissue of origin (Daussy et al., 2014; Klose et al., 2014; Peng et al., 2013). It is possible that the endogenous *Eomes* locus might be silenced in developing hILC1s after lineage specification, which is consistent with the observation that few

hILC1s appear to activate Eomes transcription in Eomes reporter mice (Paley et al., 2013). Eomes expression would, therefore, be selectively induced during cNK cell but not hILC1 maturation by upstream factors involved in early stages of ILC development. Our findings demonstrate that T-bet, a negative regulator of Eomes (Daussy et al., 2014; Gordon et al., 2012), is sufficient to repress Eomes. It is possible that T-bet might participate in silencing the *Eomes* locus in developing hILC1s, which is consistent with our observation that hILC1s appear to be stabilized in tissues that support T-bet expression. Our adoptive transfer experiments revealed that hILC1s cannot upregulate Eomes even when they repress T-bet, suggesting that T-bet might not directly inhibit Eomes expression in hILC1s. It has previously been suggested that T-bet suppresses an endogenous Th2 cell-associated program during Th1 cell responses by recruiting histone-modifying enzymes to generate repressive chromatin modifications (Zhu et al., 2012). T-bet might, therefore, employ a similar mechanism to help silence the *Eomes* locus in hILC1s.

The transcription factor Runx3 is another candidate regulator of Eomes expression. Runx3 expression is detectable in NKPs and subsequently in developing Eomes^{+} cNK cells (Levanon et al., 2014). Loss of Runx3 appears to impair the terminal maturation of cNK cells (Levanon et al., 2014), but the effect of Runx3 deficiency on Eomes expression has yet to be investigated. Runx3 is necessary for Eomes induction during CD8^{+} T cell effector differentiation (Cruz-Guilloty et al., 2009) and could possibly assume a similar role in the innate counterparts of CD8^{+} T cells.

Potential targets of T-bet and Eomes

T-bet and Eomes are structurally homologous transcription factors that can compensate for each other's functions during type 1 ILC development. Previous ChIP studies in CD8^{+} T cells and

cNK cells showed that T-bet and Eomes directly bind to the gene loci encoding IFN-γ, perforin, and granzyme B (Intlekofer et al., 2005; Townsend et al., 2004). T-bet and Eomes also assume non-redundant roles in hILC1 and cNK cell development, respectively, and it remains unclear how T-bet and Eomes might execute distinct functions during type 1 ILC development. Future ChIP studies that identify gene loci differentially targeted by T-bet and Eomes will help address this question.

Previous data from our lab suggest that T-bet directly or indirectly regulates expression of CD127, CD49a, TRAIL, and CXCR6, which are markers expressed in hILC1s (Klose et al., 2014; Paust et al., 2010; Peng et al., 2013; Takeda et al., 2005) and, to some extent, in immature cNK cells (Di Santo, 2006; Gasteiger et al., 2013; Gordon et al., 2012). Furthermore, our study suggests that Eomes appears to be upstream of CD127, CD49a, TRAIL, and integrin α_v repression, in agreement with a previous report (Gordon et al., 2012). ChIP analyses in human embryonic cells during endoderm differentiation revealed that Eomes directly binds to loci encoding CD49a and integrin α_v (Teo et al., 2011). Therefore, Eomes appears to promote cNK cell maturation by repressing markers associated with an immature cNK cell or an alternative hILC1-like fate. In support of this notion, our findings suggest that enforced Eomes expression is repressive to CD49a and TRAIL expression in type 1 ILCs from some organs, although expression of CD127 and integrin α_v was not significantly affected. Enforced T-bet expression, furthermore, was not sufficient to induce expression of CD127, CD49a, TRAIL, and integrin α_v among type 1 ILCs, suggesting that T-bet might have an indirect role in the expression of helper-like markers.

Previous studies suggested that Eomes is necessary for Ly49 receptor expression in cNK cells, while T-bet appears to be dispensable for Ly49 receptor maturation (Gordon et al., 2012; Townsend et al., 2004). Ly49 receptor genes have been proposed as putative targets of Eomes but not T-bet. In agreement with a previous report (Gordon et al., 2012), our study demonstrated that Eomes is not required for the heritable maintenance of Ly49 receptors, which suggests that Eomes does not directly target Ly49 gene loci and might recruit histone-modifying enzymes to establish heritable Ly49 gene activity. Alternatively, it is possible that Ly49 receptor expression could be supported by other factors after acute loss of Eomes. GATA-3, Ets-1, and PU.1 are necessary for induction of Ly49D, while Ets-1 additionally controls expression of Ly49H (Colucci et al., 2001; Ramirez et al., 2012; Samson et al., 2003). PU.1 and TCF-1 are also required for acquisition of Ly49D and Ly49A (Colucci et al., 2001; Held et al., 1999). TCF-1 directly binds to Ly49A promoter elements, but it is not yet known whether GATA-3, PU.1, and Ets-1 directly target Ly49 gene loci. If other factors support Ly49 receptor expression in the absence of Eomes, then one might predict that their expression would remain unchanged after Eomes is temporally deleted.

Can T-bet and Eomes expression in early ILC progenitors affect lineage specification?

We investigated whether hILC1s can undergo plasticity to a cNK cell fate using transgenic mice in which *Tbx21* locus control elements drive expression of Eomes codons. Because T-bet is not expressed at substantial levels until type 1 ILCs acquire the lineage antigens NK1.1 and NKp46 (Gordon et al., 2012; Townsend et al., 2004), we examined the effect of transgenic Eomes expression following divergent lineage specification of hILC1s and cNK cells from separate NK1.1^{-}NKp46^{-} progenitors. Transcription factors implicated in early ILC development are

currently being elucidated and we now have the tools to enforce T-bet and Eomes expression in early progenitors to all ILC lineages. To determine if T-box factors are sufficient to promote type 1 ILC lineage specification, we can generate transgenic mice in which *Nfil3*, *Tox*, or *Tcf-1* locus control elements drive expression of T-bet or Eomes codons. Transgenic mice can be bred onto an Id2 reporter background to facilitate identification of progenitor populations (Jones-Mason et al., 2012). If early T-bet or Eomes expression could divert pan-ILC progenitors toward an hILC1 or cNK cell fate, respectively, transgenic mice might have an increased representation of $Id2^+$ progenitors and hILC1s or $Id2^-$ progenitors and cNK cells.

Eomes partially accelerates cNK cell maturation during ontogeny

Mouse ontogeny is characterized by delayed onset of cNK cell development, but the basis for this delay remains incompletely understand. cNK cells represent a minor population in fetal and early neonatal type 1 ILC compartments, which are predominated by hILC1s (Gordon et al., 2012; Kubota et al., 1999; Takeda et al., 2005). Absence of substantial Ly49 receptor expression in postnatal type 1 ILCs has been attributed to a restriction on Eomes induction during early hematopoiesis in the liver (Gordon et al., 2012). Emergence of $Ly49^+$ cNK cells correlates with the shift in hematopoiesis from the liver to the bone marrow after birth. Developing type 1 ILCs in fetal and early postnatal mice do not progress well to Eomes expression, which might limit induction of Ly49 receptors.

Using transgenic mice that express Eomes when and where T-bet is expressed, we investigated whether enforced Eomes expression during early life might result in precocious cNK cell maturation. By enforcing Eomes expression when and where it is normally prohibited, we can

determine whether premature expression of Eomes and/or Ly49 receptors might be disadvantageous to the fetus. Fetal cells express a full complement of maternal and paternal MHC molecules. It is possible that early induction of MHC-I-specific Ly49 receptors might result in the activation or tolerization of fetal NK cells upon interaction with maternal cells that are "missing" paternal MHC-I molecules (Sun and Lanier, 2008b; Tripathy et al., 2008). Analysis of CD49a and CD49b (DX5) expression in type 1 ILCs revealed that transgenic neonates exhibit precocious development of $DX5^{+}$ type 1 ILCs. Type 1 ILCs in both transgenic and WT neonates, however, lacked substantial Ly49 receptor expression (Appendix, Figure A1A), suggesting that transgenic $DX5^{+}$ type 1 ILCs in neonates did not completely resemble bona fide cNK cells. Type 1 ILCs in *Tbx21*$^{-/-}$ neonates also lacked substantial Ly49 receptors, despite expressing higher than normal levels of Eomes (Appendix, Figure A1B). Overriding the ontogenic restriction on Eomes expression is, therefore, not sufficient to promote acquisition of Ly49 receptors. In an analogous manner, $Id2^{hi}$ ILCs that appear to have arisen from the helper lineage did not acquire Ly49 receptors in adult transgenic mice.

We observed that pups from a high copy number transgenic line were born at less than the expected Mendelian frequency, suggesting that early Eomes expression might result in fetal demise. Because Eomes is critical during mammalian embryogenesis (Russ et al., 2000), transgenic pups might be born less frequently due to a lethal phenotype during embryogenesis. If fetal demise were associated with precocious cNK cell development, then elimination of cNK cell development would be expected to rescue the birth rate of transgenic pups. Two different approaches could be used to disrupt type 1 ILC development in transgenic mice. One can breed transgenic mice onto a background of germline T-bet deficiency and either hematopoietic or

NKp46-specific Eomes deficiency (*Eomes*$^{flox/flox}$ *NKp46-Cre*$^{+}$ *Tbx21*$^{-/-}$ or *Eomes*$^{flox/flox}$ *Vav-Cre*$^{+}$ *Tbx21*$^{-/-}$). Alternatively, transgenic mice can be bred onto a background of common cytokine receptor γ-chain and Rag2 deficiency (*Il2rg*$^{-/-}$ *Rag2*$^{-/-}$), which would impair development of transgenic B cells and T cells in addition to type 1 ILCs.

Eomes expression might not be sufficient to expedite bona fide cNK cell development during ontogeny for a number of reasons. Firstly, recent studies have identified progenitors to all helper ILC lineages (CHILPs) in the fetal liver and intestine, but it has yet to be determined whether cNK cell progenitors (NKPs) exist in the fetus. Fetal liver progenitors are marked by expression of the transcription factors Id2 and PLZF (Constantinides et al., 2014; Klose et al., 2014), while fetal intestinal progenitors can be identified by expression of arginase-1 (Bando et al., 2015). Our study suggests that the descendant lineages of Id2^{hi} CHILPs and Id2^{-} NKPs, Eomes^{-} hILC1s versus Eomes^{+} cNK cells, continue to express high versus intermediate levels of Id2, respectively, in adults and neonates. Neonatal cNK cells, however, had higher Id2 expression than adult cNK cells. Therefore, neonatal cNK cells might descend from NKPs that express higher levels of Id2, while adult cNK cells originate from NKPs with lower or negligible Id2 expression. It is possible that Id2 expression in NKPs might vary at different times during development, which is why it will be important to determine how progenitor populations compare during early fetal/neonatal versus adult life. Future analyses of WT and transgenic pups at serial time points before birth will help clarify if and when NKPs and transgenic DX5^{+} type 1 ILCs develop in utero. If transgenic DX5^{+} type 1 ILCs develop in the absence of a clear NKP population, one might consider the possibility that Eomes diverts descendants of fetal CHILPs into a cNK cell-like fate, but is not sufficient to convert all attributes of hILC1s to those of cNK

cells. If NKPs are present in the fetus, it will be critical to investigate why their differentiation appears to be halted in the fetal milieu.

Secondly, Eomes may not be sufficient to induce all cNK cell properties in utero because systemic factors might curb the maturation of cNK cell receptors. The immunosuppressive cytokine TGF-β acts as a negative regulator of cNK cell development and maturation, contributing to cNK cell immaturity during ontogeny (Marcoe et al., 2012). Neonatal cNK cells that are resistant to TGF-β signaling progress to $CD11b^{+}$ $CD43^{+}$ stages of terminal maturation to a greater degree than their WT counterparts (Marcoe et al., 2012). Ly49 receptor acquisition, however, did not appear to be expedited in the absence of TGF-β signaling. Another group identified immunosuppressive erythroid cells that compromise host defense against perinatal pathogens in neonatal mice (Elahi et al., 2013). Due to the presence of immunosuppressive erythroid cells in the neonatal corpus, TNF-α production by adult splenocytes was compromised after co-culture with neonatal splenocytes or adoptive transfer into neonatal mice (Elahi et al., 2013). The effect of immunosuppressive erythroid cells on cNK cell maturation was not explicitly examined. Specific factors that might inhibit Ly49 receptor maturation in the fetal/neonatal corpus warrant further investigation. From present findings in the field, it is apparent that the fetal/neonatal immune system may be underdeveloped due to systemic factors, rather than cell-intrinsic defects in immune cells (Elahi et al., 2013; Marcoe et al., 2012).

Finally, liver hematopoiesis in the fetus might not support Ly49 receptor maturation in the presence of Eomes due to the absence of critical cues. Signals that are permissive to Ly49 receptor acquisition during bone marrow development might be missing in the hepatic

environment. To determine if bone marrow signals are necessary for Ly49 receptor maturation, we examined the cNK cell phenotype in mice whose cNK cell development is disrupted in the bone marrow. Because CXCR4 expression promotes NK cell localization in the bone marrow (Bernardini et al., 2013), we generated mice with a genetic deletion of CXCR4 in NKp46-expressing ILCs to impair retention of developing cNK cells in the bone marrow. A previous study suggested that CXCR4 is required for all stages of cNK development, including the progenitor stage (Noda et al., 2011). In agreement with this study, genetic deletion of CXCR4 in $NKp46^{+}$ ILCs resulted in reduction of cNK cells (Appendix, Figure A2A). However, Ly49 receptor expression in remnant cNK cells was not substantially affected, suggesting that bone marrow retention might not be critical for Ly49 receptor acquisition (Appendix, Figure A2A). Developing cNK cells acquire NKp46 before upregulating Ly49 receptors (Kim et al., 2002; Narni-Mancinelli et al., 2011) and it is, therefore, not likely that cNK cells expressed Ly49 receptors prior to deletion of CXCR4.

Using mice with genetic deletion of tumor necrosis factor-related activation-induced cytokine (TRANCE encoded by the *Tnfsf11* gene), we additionally investigated whether cNK cells acquire Ly49 receptors when bone marrow development is severely disrupted by osteopetrosis (Hsu et al., 1999; Kim et al., 2000). Examination of the liver and spleen of osteopetrotic mice revealed a reduction in cNK cells, while expression of Ly49 receptors did not appear to be impaired (Appendix, Figure A2B). Ly49 receptor maturation might, therefore, be accommodated in extramedullary sites, but it remains to be determined precisely how and where this process occurs.

The challenge of distinguishing between helper ILC1 and cNK cell lineages across tissues

Based on studies of hepatic type 1 ILCs, it has been suggested that hILC1 and cNK cell lineages are distinguishable by mutually exclusive expression of integrins CD49a and CD49b (Daussy et al., 2014; Peng et al., 2013; Sojka et al., 2014). Microarray analyses revealed that CD49a appears to be the most specific marker of hepatic hILC1s (Daussy et al., 2014; Peng et al., 2013). The distinction between hILC1 and cNK cell lineages in extra-hepatic tissues, however, remains more challenging. Consistent with prior reports (Cortez et al., 2014; Sojka et al., 2014), the findings in this study suggest that $CD49a^+$ cells in extra-hepatic tissues differ from their hepatic counterparts and appear to exhibit some properties of cNK cells. Although $CD49a^+$ $DX5^-$ cells in the uterus are sessile (Sojka et al., 2014), they have abundant expression of Eomes and Ly49 receptors. A subset of $CD49a^+$ cells in the salivary gland and thymus, moreover, express CD49b, Eomes, and Ly49 receptors. "Hybrid" type 1 ILC subsets might represent a third lineage or a putative intermediate of cNK cell development and these possibilities can be reconciled in future adoptive transfer experiments. Identification of hILC1s in extra-hepatic tissues is further complicated by the possibility that CD49a may not be expressed in all $Eomes^-$ type 1 ILCs. hILC1 markers might also be insufficient to discriminate between $Eomes^-$ hILC1s and $Eomes^+$ cNK cells (Robinette et al., 2015). Furthermore, Eomes might not be genetically upstream of CD49a repression in non-lymphoid organs where Eomes appears insufficient to repress CD49a expression. Differences in the development and phenotype of tissue-specific type 1 ILC subsets complicate the identification of markers that can reliably differentiate between hILC1 and cNK cell lineages across all tissues.

Based on our findings, it is possible that an unambiguous distinction between hILC1s and cNK cells across tissues can be made on the basis of Id2 and Eomes expression. Id2 correlates negatively with Eomes expression in type 1 ILCs, such that high Id2 expression marks Eomes^{-} hILC1s while intermediate Id2 levels are associated with Eomes^{+} cNK cells. In future studies, it will be helpful to examine gene expression in hILC1 and cNK cell subsets distinguished according to Id2 and Eomes expression in mice with dual Id2 (Jones-Mason et al., 2012) and Eomes (Daussy et al., 2014) reporter activity. Transcriptional data generated using dual Id2/Eomes reporter will be useful for comparison with data from a recent study of ILC gene expression profiles (Robinette et al., 2015).

Perspective

Type 1 ILCs contribute to immunosurveillance against numerous pathogens and cancer, which is why it is important to understand how they develop in lymphoid and non-lymphoid tissues. Type 1 ILCs consist of distinct hILC1 and cNK cell populations that develop from separate lineages and appear to be suited for unique functions in host immunity. The relationship between hILC1s and cNK cells subsequent to their divergent lineage specification had not been closely examined in previous studies. This study revealed that type 1 ILCs with distinct lineage origins have the potential for substantial developmental plasticity, in an analogous manner to T cell lineages. Future studies will help uncover signals both upstream and downstream of T-box factors that contribute to fate determination of type 1 ILCs. Consideration of how environmental factors influence the ontogeny of type 1 ILCs could help justify differences in the development of tissue-specific subsets, in addition to offering an explanation for why the early immune system appears to be underdeveloped. Enhanced knowledge of the cell-intrinsic and cell-extrinsic cues

that regulate type 1 ILC development and plasticity might help usher in new strategies for type 1 ILC-based therapies against infection and cancer.

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APPENDIX

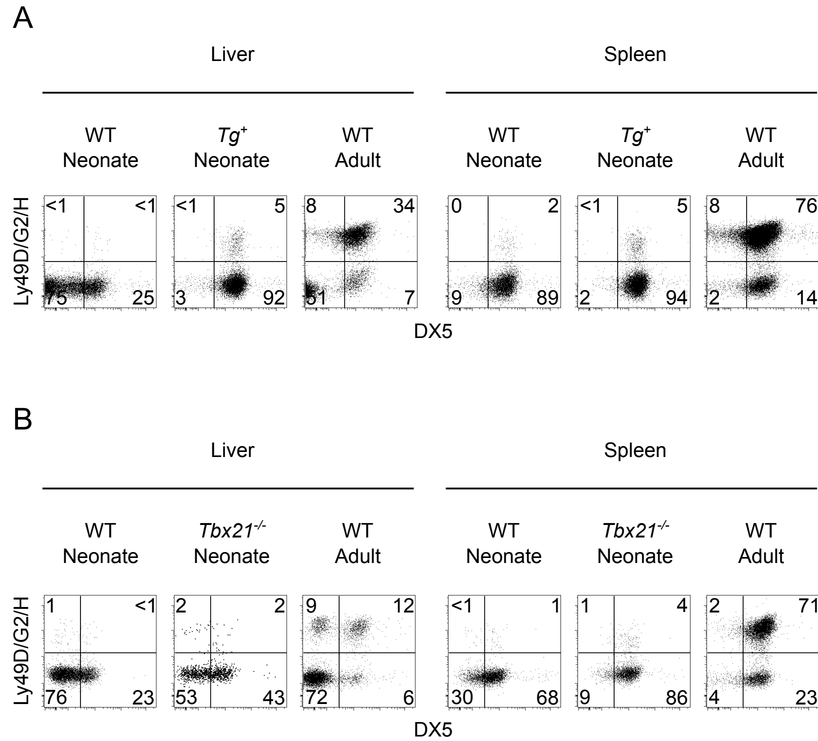

Figure A1. Eomes cannot override the restriction on Ly49 receptor expression during ontogeny. Ly49 and CD49b expression by NK1.1$^+$ NKp46$^+$ type 1 ILCs from the livers and spleens of (**A**) WT neonates, *Tg*$^+$ neonates, and WT adults (n=4 mice per genotype), and (**B**) WT neonates, *Tbx21*$^{-/-}$ neonates, and WT adults (n=3 mice per genotype). Data are representative of 3–4 independent analyses.

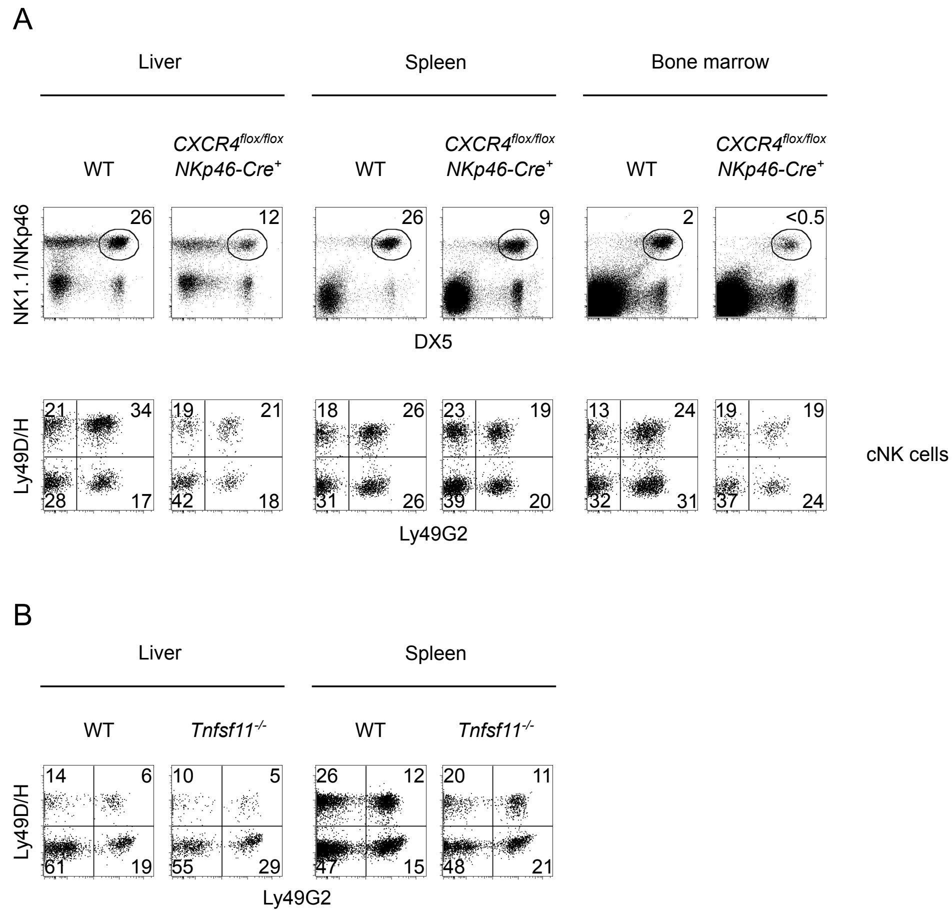

Figure A2. Ly49 receptor expression is supported in extramedullary tissues.
(**A**) Flow cytometry showing the frequency of NK1.1^{+} NKp46^{+} DX5^{+} cNK cells among Lin (CD3, Gr-1, TER-119, CD19)$^{-}$ cells from the livers and spleens of WT and *CXCR4*$^{flox/flox}$ *NKp46-Cre*$^{+}$ mice. Plots also illustrate Ly49 receptor expression by cNK cells (n=2 mice per genotype). (**B**) Flow cytometry of Ly49 receptor expression by cNK cells from the livers and spleens of WT and *Tnfsf11*$^{-/-}$ mice (n=3 mice per genotype). Data are representative of 2–3 independent analyses.