DEVELOPMENTAL CONTROL OF INNATE LYMPHOID CELLS

By
Theresa L. Geiger

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Joseph Sun, PhD Dissertation Mentor	Date

Dedication

To my family, for all of their love and support

“A loveless world is a dead world, and always there comes an hour when one is weary of prisons, of one's work, and of devotion to duty, and all one craves for is a loved face, the warmth and wonder of a loving heart.”

- Albert Camus, *The Plague* -

Abstract

Innate lymphoid cells (ILCs) are a recently defined lineage of cells derived from the common lymphoid progenitor (CLP). They are primarily found at barrier surfaces such as the intestine and skin, and have established roles in homeostasis and protection from pathogens at these sites through their production of cytokines. The founding member of the ILC lineage, natural killer (NK) cells, can also rapidly kill infected or transformed cells. Here, we report on two different players in the complex network of transcription factors and cytokines that govern ILC development: the transcription factor Nfil3 and the cytokine TGF-β. We show that the transcription factor Nfil3 is not only required for the development of NK cells, but also all other ILC lineages. Nfil3-deficient mice lack all ILC subsets, including NK cells, and are severely deficient in an early ILC progenitor. Nfil3-deficient mice are also drastically more susceptible to intestinal pathogens such as *C. rodentium* and *C. difficile*, likely due to their lack of intestinal ILCs. Suprisingly, *Nfil3*$^{-/-}$ mice are only slightly more susceptible to chemically induced colitis and are less susceptible to a carcinogen-induced model of colorectal cancer, indicating ILCs may also play a pathogenic role in certain settings. Furthermore, we show that the cytokine TGF-β is dispensable for normal NK cell development. Deletion of TGF-β signaling in NK cells at the NK progenitor (NKP) stage has no observed affect on NK cell development. However, TGF-β insensitive NK cells appear to have a defect in expansion and persistence after viral infection, indicating that TGF-β may play a role in determining effector and memory NK cell fates. These findings have increased

our understanding of the complex network of signals governing ILC development. In particular, our identification of Nfil3 as a transcription factor necessary for both NK cell and ILC development helps establish the relationship between these two lineages during early lymphoid development.

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List of Abbreviations

AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
AOM	azoxymethane
Areg	amphiregulin
Arg1	arginase-1
BATF3	basic leucine zipper transcription factor ATF-like 3
Bcl-2	B-cell lymphoma 2
Bcl11b	B-cell CLL/lymphoma 11b
Blimp1	B-lymphocyte-induced maturation protein 1
BM	bone marrow
BMP	bone morphogenic protein
BMT	bone marrow transplant
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3 ligand
BrdU	5-Bromo-2´-deoxyuridine
bZIP	basic leucine zipper
C/EBP	CCAAT/enhancer binding protein
Cbfβ	core binding factor β
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CFU	colony forming units
CHILP	common helper innate lymphoid cell progenitor
CLP	common lymphoid progenitor
co-Smad	common mediator Smad
CXCR	CXC chemokine receptor
DAI	disease activity index
DC	dendritic cell
DKO	double knockout

DLL	delta-like ligand
DNA	deoxyribonucleic acid
DSS	dextran sulfate sodium
Ebf-1	early B cell factor 1
EILP	early innate lymphoid cell progenitor
Eomes	eomesodermin
FBS	fetal bovine serum
FLICA	fluorochrome-labeled inhibitors of caspases
Flt-3	Fms-related tyrosine kinase 3
Gfi1	growth factor independent 1 transcription repressor
GSK	Gerstner Sloan Kettering
GVHD	graft-versus-host disease
HCMV	human cytomegalovirus
HSC	hematopoietic stem cell
I.P.	intraperitoneal
I.V.	intravenous
IACUC	institutional animal care and use committee
IBD	inflammatory bowel disease
Id	inhibitor of DNA binding
IFN	interferon
IL	interleukin
IL-10R	interleukin 10 receptor
IL-12R	interleukin 12 receptor
IL-2Rγc	interleukin 2 receptor γ chain
IL-7Rα	interleukin 7 receptor α
ILC	innate lymphoid cell
ILCP	innate lymphoid cell precursor
iNK	immature natural killer
Irf2	interferon regulatory factor 2

JAK	Janus kinase
KLRG1	killer cell lectin-like receptor G1
Lamp1	lysosomal-associated membrane protein 1
LAP	latency associated peptide
LCMV	lymphocytic choriomenigitis virus
LI	large intestine
LTi	lymphoid tissue inducer
LTPB	latent transforming growth factor β binding protein
MAPK	mitogen-activated protein kinase
MCMV	mouse cytomegalovirus
MEF	monocyte enhancer factor
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MITF	microphthalmia-associated transcription factor
mLN	mesenteric lymph node
mNK	mature natural killer
MPEC	memory precursor effector cell
MPP	multi-potent progenitor
MSKCC	Memorial Sloan Kettering Cancer Center
mTOR	mammalian target of rapamycin
Myd88	myeloid differentiation primary response gene 88
Nfil3	nuclear factor IL-3 regulated
NK	natural killer
NKP	natural killer cell progenitor
PAR	proline and acid rich
Pax5	paired box 5
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	phosphoinositide dependent protein kinase

PFU	plaque forming units
PI	post-infection
PI3K	phosphoinositide 3-kinase
PLZF	promyelocytic leukemia zinc finger protein
PP	Peyer's patches
qRT-PCR	quantitative real-time polymerase chain reaction
R-Smad	receptor regulated Smad
RAG	recombination-activating gene
RNA	ribonucleic acid
ROR	retinoic acid receptor-related orphan receptor
Runx3	runt-related transcription factor 3
S1P5	Sphingosine-1-phosphate receptor 5
Sca-1	stem cell antigen-1
SD	standard deviation
SI	small intestine
SLEC	short-lived effector cell
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducer and activator of transcription
Tcf-1	T-cell factor 1
TCR	T cell receptor
TGF-β	transforming growth factor β
TGF-βR	transforming growth factor β receptor
T_h	T helper (cell)
Tox	thymocyte selection-associated high mobility group box
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VAT	visceral adipose tissue
VRE	vancomycin-resistant *Enterococcus faecium*
Zbtb32	zinc finger and BTB domain containing 32

Chapter 1: Introduction

I. Development of Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are a critical component of the immune system, best known for their ability to rapidly produce cytokines upon stimulation. They are the third lineage of lymphocyte derived from the common lymphoid progenitor (CLP), along with the adaptive lymphocytes, B and T cells. However, unlike B and T cells, they do not undergo antigen receptor gene rearrangement mediated by the RAG recombinase (Spits and Cupedo 2012). The founding member of this diverse family of lymphocyte is the natural killer (NK) cell, known for its ability to produce cytotoxic molecules (granzymes and perforin) as well as the cytokine interferon (IFN)-γ (Kiessling, Klein, Pross, et al. 1975; Kiessling, Klein and Wigzell 1975). The family has recently been expanded to include group 1, group 2, and group 3 ILCs (Figure 1) (Spits et al. 2013). Group 1 ILCs include conventional NK cells as well as non-NK cell ILC1, which produce IFN-γ but are not thought to mediate cytotoxicity (Klose et al. 2014; Bernink et al. 2013; Fuchs et al. 2013). Both NK cells and ILC1 express the transcription factor T-bet (Seillet et al. 2015). Group 2 ILCs produce interleukin (IL)-13 and IL-5, and express the transcription factor Gata-3 (Moro et al. 2010; Neill et al. 2010; Price et al. 2010). Group 3 ILCs include lymphoid tissue inducer cells (LTi) as well as non-LTi ILC3. LTis are important for the development of lymphoid organs such as lymph nodes and Peyer's patches (Mebius et al. 1997). Group 3 ILCs produce IL-22 and IL-17 and express the transcription factor ROR-γt (Satoh-Takayama et al. 2008; Eberl

et al. 2004; Sun et al. 2000). The diversity among ILCs mirrors the diversity among T cells, with NK cells sharing transcriptional and functional characteristics with cytotoxic $CD8^+$ T cells, IFN-γ producing ILC1 with T_h1 cells, type 2 cytokine-producing ILC2 with T_h2 cells, and IL-22 and IL-17 producing ILC3 with T_h17 and T_h22 cells (Figure 2) (Huntington et al. 2016). ILCs can be found in various lymphoid and non-lymphoid organs, with NK cells circulating throughout the vasculature and found in all organs, and other ILCs being tissue-resident (Gasteiger et al. 2015). ILC1 are primarily resident in the liver, uterus, salivary glands, and intestines (D K Sojka et al. 2014; Cortez et al. 2014; Kim et al. 2016), while ILC2 are most prevalent in the skin, fat, and large intestine, and ILC3 are most prevalent in the intestines (Figure 3) (Kim et al. 2016). We shall hereafter use the terminology “group 1 ILCs” to include both NK cells and non-NK cell ILC1, and “group 3 ILCs” to include ILC3 and LTi cells (Figure 1).

1. ILC Precursors

ILCs are a lymphocyte lineage derived from the CLP. The first committed ILC precursor (ILCP) downstream of the CLP is a $CD127^+$ a4β7 integrin-expressing cell that gives rise to NK cells, dendritic cells, and LTi cells but not B or T cells (Yoshida et al. 2001). Similarly, an $α4β7^+$ IL-$7Rα^+$ $CXCR6^+$ subset of the CLP has been shown to have ILC3 and NK cell but not T or B cell lineage potential (Possot et al. 2011); this subset is now commonly referred to as the αLP (Figure 4) (X. Yu et al. 2014; Serafini et al. 2015). Consistent with their constitutive expression of IL-7Rα, all ILCs depend on IL-7 and the IL-2 receptor common γ chain for their development (Spits and Di Santo 2011; Spits and

Cupedo 2012). We were one of several groups to first identify the importance of the transcription factor Nfil3 (E4bp4) in the development of all ILC lineages, including NK cells and LTi cells (Geiger et al. 2014; Seillet, Rankin, et al. 2014; X. Yu et al. 2014; Xu et al. 2015). This discovery will be discussed in detail in Chapter 2. Briefly, Nfil3 is now thought to be required for the transition from the CLP to the αLP (Figure 4). ILC defects in *Nfil3*$^{-/-}$ bone marrow cells can be rescued by retroviral transduction with vectors expressing the transcription factor Tox, suggesting that Tox is also involved in early ILC development downstream of Nfil3 (X. Yu et al. 2014). Tox had been previously shown to be required for the development of NK cells and LTi cells (Aliahmad et al. 2010; Vong et al. 2014) and more recently, it has been shown that Tox is indeed required for the transition from the CLP to a shared NK cell/ILC precursor (Seehus et al. 2015). Similarly, a precursor capable of developing into all ILC lineages termed the early ILC progenitor or "EILP" has been identified by expression of the transcription factor Tcf-1 (Q. Yang et al. 2015). Tcf-1 is required for generation of all ILC lineages, as Tcf-1 deficient mice have severe defects in all ILCs (Q. Yang et al. 2015). Interestingly, a novel arginase 1 (Arg1)-expressing ILC progenitor has also been identified in the fetal intestine, and may be responsible for seeding that organ of ILCs during fetal development (Bando et al. 2015).

2. Differentiation of ILC1 and NK cells

The defining transcription factor for group 1 ILCs (including ILC1 and NK cells) is T-bet. T-bet deficient mice lack ILC1s and have a defect in terminal maturation of NK cells (Gordon et al. 2012; Klose et al. 2014). T-bet and

downstream transcription factor Runx3 are critical for the production of IFN-γ, a hallmark of group 1 ILCs (Djuretic et al. 2007), and Runx3 has been further shown to be required for ILC1 devlopment and maintenance (Ebihara et al. 2015). NK cells can be distinguished from ILC1 by their expression of the transcription factor Eomes and the surface marker CD49b (DX5) (Seillet et al. 2015). In addition, forced overexpression of Eomes can drive ILC1s into an NK cell-like fate (Pikovskaya et al. 2016). Conventional NK cells are also thought to recirculate through the vasculature, whereas ILC1 are thought to be tissue resident (Gasteiger et al. 2015). These findings indicate that previously identified liver- and thymic-resident NK cells (Takeda et al. 2005; Seillet, Huntington, et al. 2014; Smyth et al. 2001; Vosshenrich et al. 2006) may in fact be ILC1, supported by recent evidence that they are transcriptionally distinct from conventional non-resident NK cells (Daussy et al. 2014; Seillet et al. 2015; Seillet, Huntington, et al. 2014; Robinette et al. 2015). Furthermore, there is some debate about the classification of non-NK cell ILC1 as group 1 ILCs based on their IFN-γ expression, as some studies indicate that they may develop from ROR-γt$^+$ ILC3 (Spits et al. 2013; Bernink et al. 2013; Klose et al. 2013; Vonarbourg et al. 2010).

The importance of PLZF in ILC differentiation was shown using PLZF reporter mice to demonstrate that PLZF$^+$ ILCPs generate all ILC lineages except NK cells and LTi cells (Figure 4) (Constantinides et al. 2014). However, PLZF deficient mice have a defect in ILC1 and ILC2 but not NK cells or group 3 ILCs (Constantinides et al. 2014), indicating that PLZF is not required for all ILCs but may play a role in promoting certain lineages over others. Recent work has

supported this idea, showing that PLZF contributes to the divergence of NK cells from ILC1 (Constantinides et al. 2015). Id2 has similarly been shown to map an early ILC precursor that does not give rise to NK cells, but does give rise to all other ILC lineages including LTi cells (Klose et al. 2014). This has led to the $Id2^{+}$ precursor being designated as the “common helper innate lymphoid cell progenitor” or CHILP (Figure 4) (Klose et al. 2014). Gata3 is another transcription factor that has been shown to be required for development of ILC1, ILC2, ILC3 and LTi cells, but not conventional NK cells (Zhong et al. 2016; Serafini et al. 2014; Yagi et al. 2014; Vosshenrich et al. 2006). These findings support the idea that NK cells are a separate lineage from the “helper” ILCs, including ILC1. Other distinct developmental requirements for ILC1 remain to be elucidated. The development of NK cells will be discussed in detail in Section II.

3. Differentiation of ILC2

Gata3 is the defining transcription factor for ILC2s. ILC2s express high levels of Gata3 and require it for development and function (Klein Wolterink et al. 2013; Hoyler et al. 2012; Yagi et al. 2014). Gata3 is particularly critical for optimal production of type 2 cytokines by ILC2s (Liang et al. 2012). The Notch target transcription factor Bcl11b is also important for ILC2 development and maintenance (Califano et al. 2015; Walker et al. 2015; Yu et al. 2015). Bcl11b-expressing ILCP generate only ILC2 (Yu et al. 2015). Furthermore, *Bcl11b*$^{-/-}$ bone marrow fails to develop into ILC2 in fetal liver chimeras (Walker et al. 2015) and ablating Bcl11b in mature cells results in a loss of ILC2 identity and function measured by Gata3 expression and responsiveness to IL-33 (Califano et al.

2015). The transcription factor ROR-α has similarly been shown to be critical for ILC2 development (Figure 4), as ROR-α-deficient mice lack ILC2 (Halim, MacLaren, et al. 2012; Wong et al. 2012). The transcription factor Gfi1 also promotes ILC2 development and function (Figure 4). *Gfi1*$^{-/-}$ bone marrow has a defect in producing ILC2 in a mixed bone marrow chimera setting (Spooner et al. 2013). Gfi1 is also thought to regulate the expression of the IL-33 receptor and thereby IL-33 responsiveness (Spooner et al. 2013).

4. Differentiation of ILC3 and LTi Cells

Group 3 ILCs are differentiated from other ILCs by their expression of and dependence upon the transcription factor ROR-γt (Eberl et al. 2004; Satoh-Takayama et al. 2008; Sanos et al. 2009; Luci et al. 2009; Vonarbourg et al. 2010). Another transcription factor important in the development of group 3 ILCs is AhR (Figure 4). *AhR*$^{-/-}$ mice were shown to have a severe reduction in NKp46^{+} ILC3 and CD4^{+} LTi cells (Lee et al. 2012). However, AhR appears to be critical only for adult, not fetal, ILC3, as *AhR*$^{-/-}$ mice develop early-developing structures such as lymph nodes and peyer's patches but lack later developing cryptopatches and lymphoid follicles (Lee et al. 2012; Kiss et al. 2011; Qiu et al. 2012). Conversely, mice lacking the transcription factor Tox lack lymph nodes and have a severe defect in peyer's patches, likely due to their lack of LTi cells (Figure 4) (Aliahmad et al. 2010). Recent work has further suggested that LTi cells may differentiate from other ILCs as early as the αLP, which may be a heterogeneous mixture of ILCPs and LTi precursors (Ishizuka et al. 2016). This supports earlier work showing that LTi cells, like NK cells, are not derived from a

PLZF^{+} precursor that generates all other ILC lineages (Constantinides et al. 2014). Runx3 has also been shown to be required for development but not maintenance of ILC3 (Ebihara et al. 2015), while Gata3 is required for both development and maintenance of ILC3 (Zhong et al. 2016).

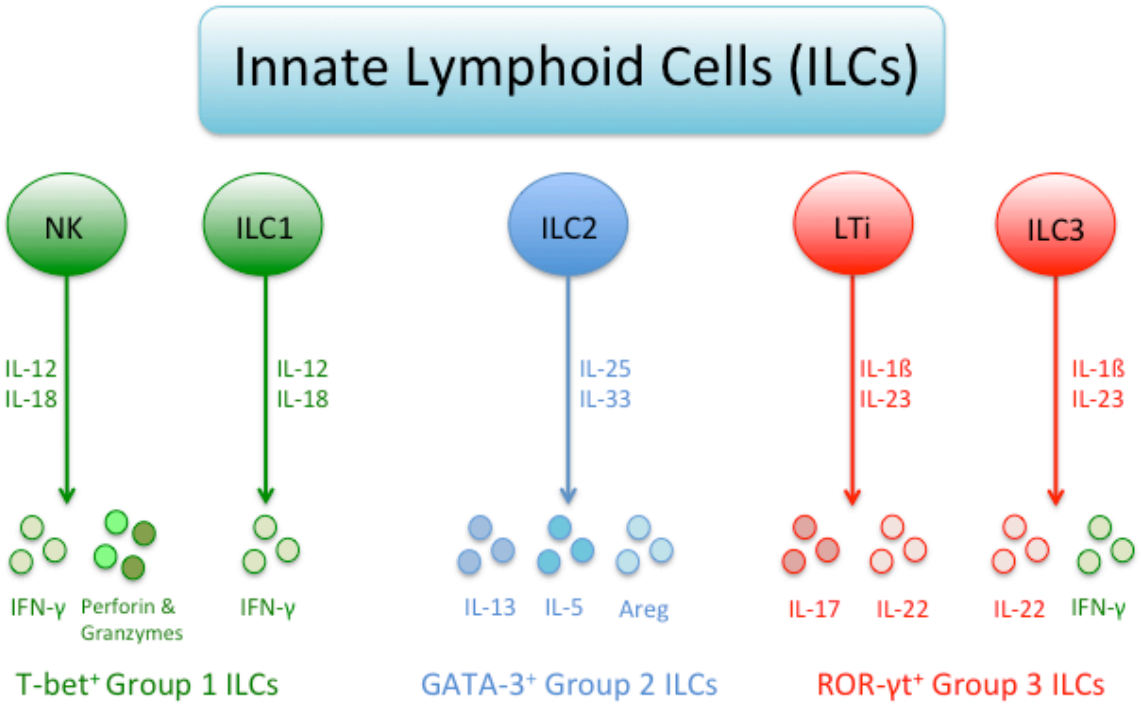

FIGURE 1 | INNATE LYMPHOID CELL SUBSETS

Group 1 ILCs (green) include classical NK cells and ILC1, and are defined by their expression of the transcription factor T-bet and their ability to produce IFN-γ upon stimulation. Group 2 ILCs (blue) or ILC2s are defined by their high expression of the transcription factor Gata-3 and their ability to produce type 2 cytokines and amphiregulin upon stimulation. Group 3 ILCs (red) include LTi cells and ILC3 and are defined by their expression of the transcription factor ROR-γt and their ability to produce IL-17 and IL-22 upon stimulation.

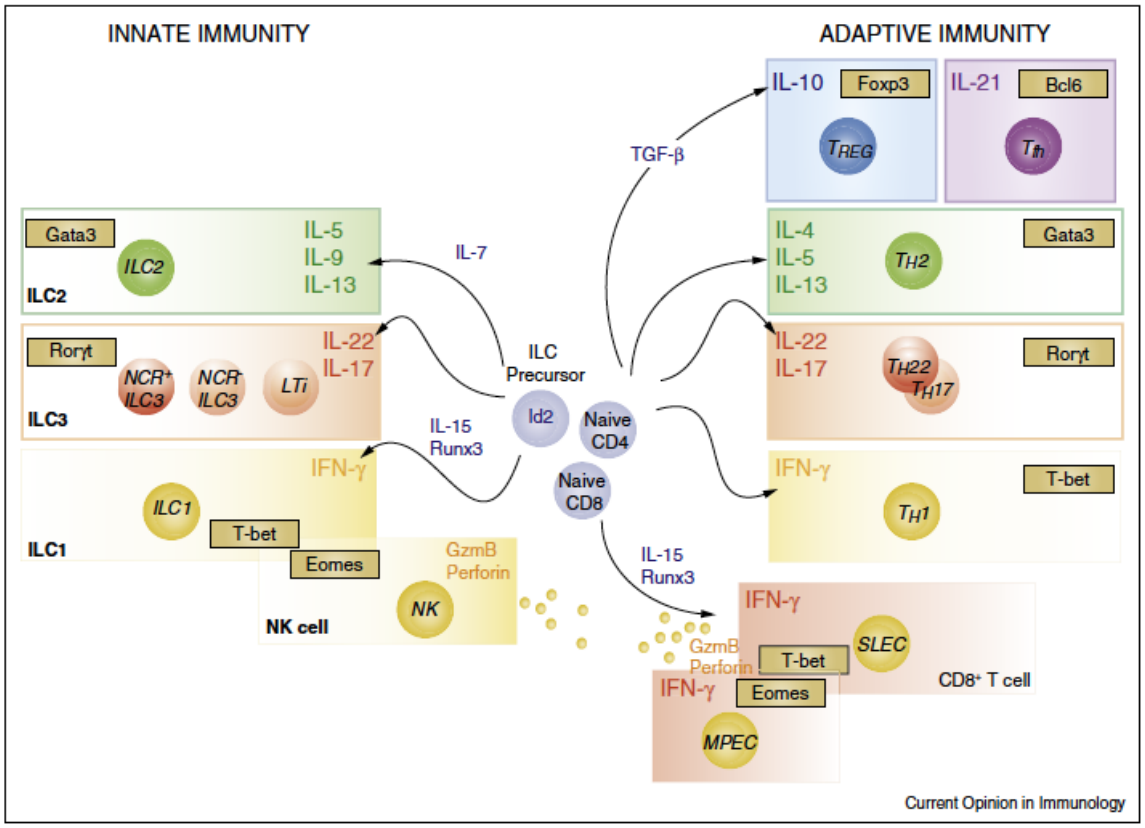

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FIGURE 2 | PARALLELS BETWEEN ILCS AND T CELLS

ILC subsets share transcriptional and functional similarities with T cell subsets. Group 1 ILCs and T_h1 cells (yellow) express T-bet and produce IFN-γ. NK cells and $CD8^+$ T cells produce lytic molecules. ILC2 and T_h2 cells (green) express Gata3 and produce type 2 cytokines. Group 3 ILCs and T_h22 and T_h17 cells (red) express ROR-γt and make IL-22 and IL-17.

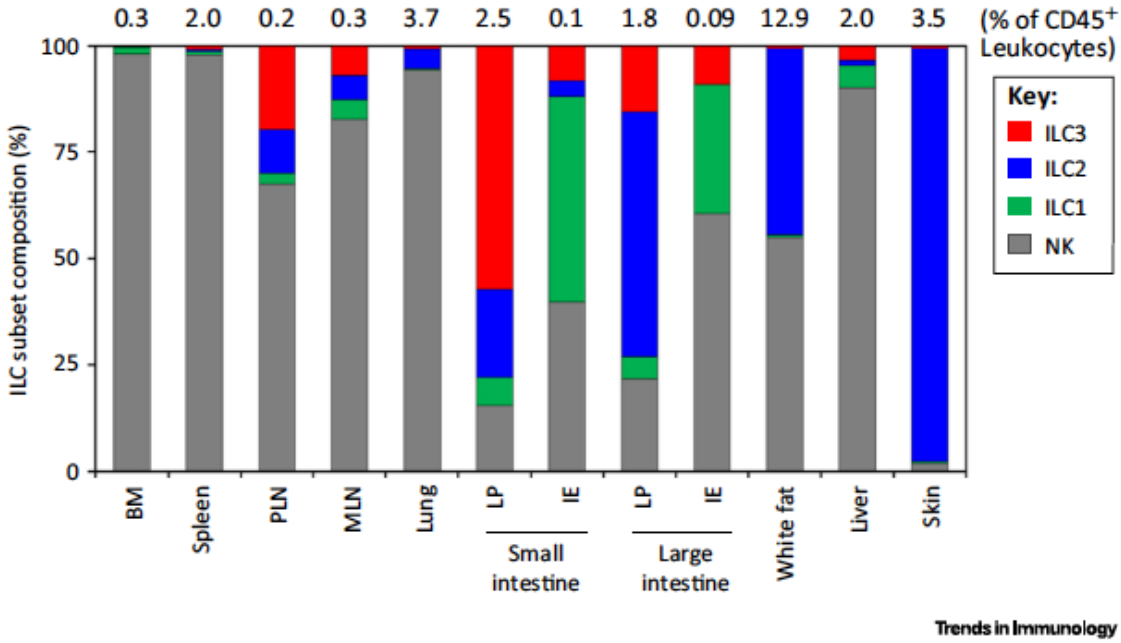

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FIGURE 3 | TISSUE LOCATIONS OF ILCS

ILCs compose varying percentages of the total $CD45^+$ lymphocyte compartment in different organs.

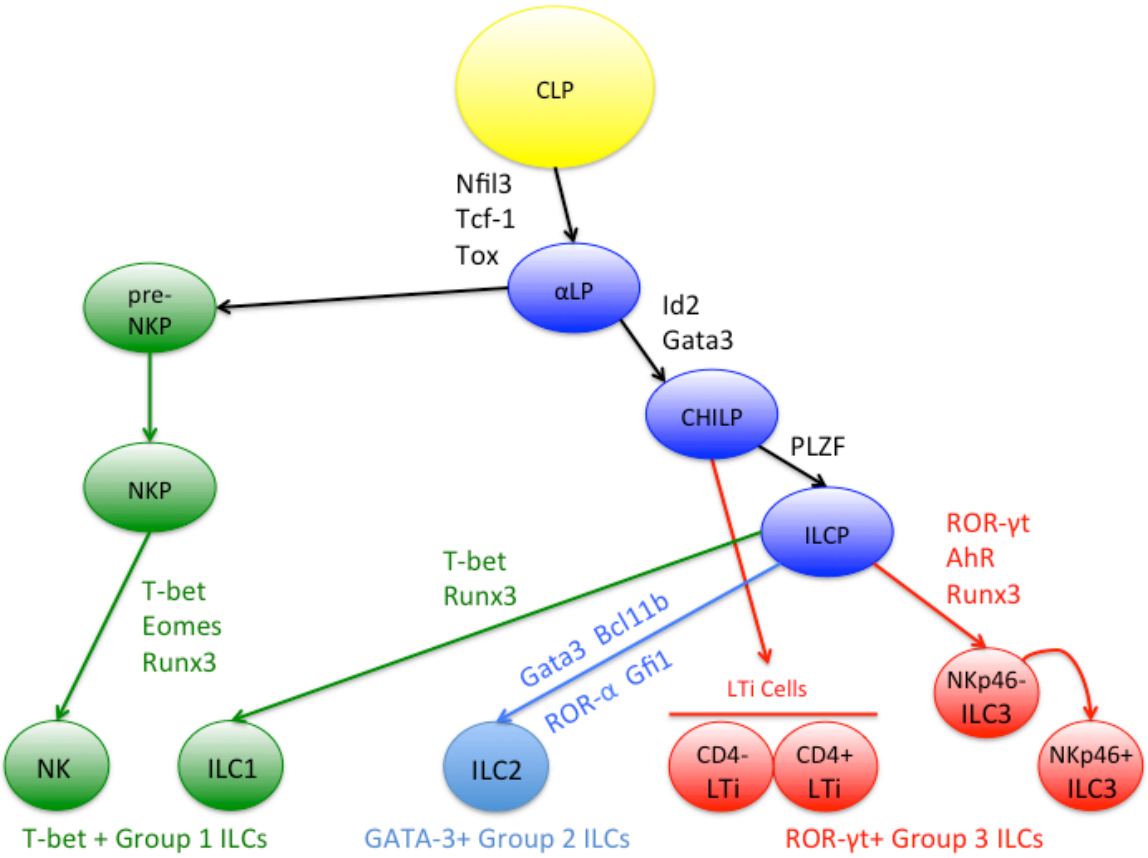

FIGURE 4 | INNATE LYMPHOID CELL DEVELOPMENT

ILCs differentiate from the CLP (yellow). Factors important early in development to a shared NK cell and helper ILC precursor (αLP) include Nfil3, Tcf-1, and Tox. Downstream of the αLP is the pre-NKP and the CHILP. Downstream factors required for differentiation into group 1 ILCs (green), group 2 ILCs (blue) and group 3 ILCs (red) are shown. A more detailed diagram of NK cell development is shown in Figure 6.

II. Development and Maturation of Natural Killer Cells[1]

NK cells, like B and T cells, are a lymphocyte lineage derived from the CLP (Kondo et al. 1997), and like B cells, are thought to develop primarily in the bone marrow (Rosmaraki et al. 2001), although other sites of development, such as the liver and thymus, have also been proposed (reviewed in (Dorothy K Sojka et al. 2014)). However, unlike the antigen receptors of B and T cells, NK cell receptors are germ line encoded and do not require gene rearrangement by RAG recombinase (Lanier et al. 1986), though recent work has suggested that RAG plays an unexpected cell-intrinsic role in NK cell development (Karo et al. 2014). NK cells also undergo an "education" process during development where they acquire the ability to recognize lack of self MHC class I, or "missing-self", a feature that facilitates their surveillance of target cells that have down-regulated MHC class I during infection or malignancy (Orr and Lanier 2010). NK cells rely on both cytokines and transcription factors to promote and control their development. Cytokine signaling from interleukin (IL)-15 is critical for the development of NK cells and is required throughout their lifetime (Di Santo 2006; Yokoyama et al. 2004). Transcription factors such as Nfil3 and PU.1 are necessary for development of early NK cell progenitors (Gascoyne et al. 2009; Kamizono et al. 2009; Kashiwada et al. 2010; Colucci et al. 2001), whereas Id2, Tox, and others are important later in development (Yokota et al. 1999; Boos et al. 2007; Aliahmad et al. 2010). Eomes and T-bet are among factors that then control the final stages of NK cell maturation (Townsend et al. 2004; Gordon et al. 2012). In the periphery, the activation and differentiation of NK cells are

regulated by a plethora of transcription factors mediating distinct effector functions. This review will outline current knowledge about the stages of NK cell development and the factors driving each stage.

1. Stages of NK Cell Development and Differentiation[1]

The CLP is characterized by expression of IL-7Rα (CD127), c-kit (CD117) Sca-1, and Flt-3 (CD135), as well as the lack of common lineage markers such as CD3, CD4, CD8, CD19, Ter119, Gr-1 and NK1.1 (Figure 5) (Kondo et al. 1997). From the CLP, cells develop into NK cell precursors (NKP), which are defined by expression of the IL-15 receptor β chain (CD122), and lack of common lineage markers, including the NK cell markers NK1.1 and DX5 (CD49b) (Figure 5) (Rosmaraki et al. 2001). This NKP population has been further refined based on the co-expression of CD27 and CD244, with the majority of these cells also expressing IL-7Rα (Fathman et al. 2011). An intermediate population between the CLP and NKP termed “pre-NKP” has also recently been defined as lineage negative, $CD244^{+}$ c-kit^{low} IL-$7R\alpha^{+}$ Flt-3^{-} and $CD122^{-}$ (Fathman et al. 2011; Carotta et al. 2011). However, recent work suggests that this population is heterogeneous, composed of true NK-committed precursors as well as PLZF- and α4β7 integrin-expressing ILC precursors (ILCP) (Figure 5) (Constantinides et al. 2015). A precursor of this pre-NKP population also capable of producing all ILC lineages (including NK cells) has recently been identified by expression of the transcription factor Tcf-1 (Q. Yang et al. 2015). From the $CD122^{+}$IL-$7R\alpha^{+/-}$ NKP stage, cells develop into immature NK (iNK) cells, which lose expression of IL-7Rα and acquire expression of NK1.1 but do not yet

express CD49b (Figure 5) (Rosmaraki et al. 2001). As immature NK cells gain expression of CD11b, CD43, Ly49 receptors, and CD49b (DX5), they also gain functional competence in cytotoxicity and production of interferon (IFN)-γ (Kim et al. 2002), and egress from the bone marrow.

The peripheral NK cell pool can be delineated by their expression of CD27, with $CD27^{lo/-}$ NK cells being more cytotoxic and producing more cytokines than $CD27^{high}$ NK cells (Hayakawa and Smyth 2006). These mature peripheral NK cell populations have more recently been further refined into four stages of maturation, defined by sequential upregulation of CD11b expression followed by downregulation of CD27, with the most immature NK cells being $CD27^{-}CD11b^{-}$ and the most mature NK cells being $CD27^{-}CD11b^{+}$ (Chiossone et al. 2009). During viral infection or pro-inflammatory cytokine exposure, mature peripheral NK cells can differentiate into effector and long-lived memory NK cells (reviewed in (Min-Oo et al. 2013)). During the $CD8^{+}$ T cell response to viral infection, at least two different effector cell populations are thought to be generated: $KLRG1^{hi}$ short-lived effector cells (SLECs) and $KLRG1^{lo}$ memory precursor effector cells (MPECs) (Kaech and Wherry 2007). Recent evidence suggests that a similar paradigm exists in the resting NK cell pool, with virus-specific $KLRG1^{-}$ NK cells exhibiting a greater capacity to generate memory NK cells than their $KLRG1^{+}$ counterparts (Kamimura and Lanier 2015). In accordance with this finding, another recent study found that RAG expression during NK cell ontogeny was correlated with lower expression of KLRG1 and a greater memory potential (Karo et al. 2014).

2. Transcriptional Control of Early NK Cell Development[1]

Lineage commitment to either an adaptive or innate lymphocyte cell fate is determined by a complex network of transcription factors (Figure 6). For example, Notch signaling through the ligands Jagged1 and Jagged2 preferentially drives NK cell development from the CLP (DeHart et al. 2005; Jaleco et al. 2001; Lehar et al. 2005), whereas delta-like ligands (DLL) promote T cell development (Maillard et al. 2005). Moreover, thymocytes can be diverted into an NK cell-like fate if the Notch1-dependent transcription factor Bcl11b is ablated during T cell development (Ikawa et al. 2010; P. Li et al. 2010; L. Li et al. 2010), suggesting active suppression of the NK cell fate. Similarly, early B cell factor 1 (Ebf1) and Pax5 promote the B cell fate by suppressing expression of ILC and T-cell promoting transcription factors Notch1, Tcf-1, Gata3, and Id2 (De Obaldia and Bhandoola 2015). Even within the innate lymphocyte lineages, differential expression of specific transcription factors give rise to distinct cell fates. For example, although both NK cells and non-NK cell "helper" ILCs require the transcription factors Id2 (Yokota et al. 1999; Moro et al. 2010; Satoh-Takayama et al. 2010) and Nfil3 (Gascoyne et al. 2009; Kamizono et al. 2009; Kashiwada, Pham, et al. 2011; Geiger et al. 2014; Seillet, Rankin, et al. 2014; Xu et al. 2015; X. Yu et al. 2014) for their development, only the helper ILC lineages require Gata3 for development (Yagi et al. 2014; Serafini et al. 2014; Zhong et al. 2016). These differential requirements are consistent with recent studies indicating that ILCs are not derived from the same $CD122^+$ precursor as NK cells, but rather arise from an IL-$7R\alpha^+$, $\alpha4\beta7^+$, Id2-expressing precursor, referred to as

the common helper innate lymphoid cell precursor or “CHILP” (reviewed in (Serafini et al. 2015)).

Nfil3 (also known as E4BP4) is a critical factor in NK cell lineage commitment. Originally identified as a circadian clock gene (Mitsui et al. 2001), Nfil3 is widely expressed in many hematopoietic and non-hematopoietic cells, and is expressed as early as the CLP stage in developing lymphocytes (Male et al. 2014). Early studies in Nfil3-deficient mice revealed a specific loss of NK cells, whereas numbers of B cells, $CD4^+$, and $CD8^+$ T cells were normal (Gascoyne et al. 2009; Kamizono et al. 2009; Kashiwada et al. 2010). Later studies revealed that Nfil3 expression is only required in developing NK cells through the NKP stage; conditional deletion during the iNK stage does not impact NK cell numbers, cytokine production, or response to viral infection (Firth et al. 2013). Nfil3 expression is thought to be driven by IL-15, as IL-15 induces Nfil3 expression in NK cells and ectopic Nfil3 expression can partially rescue NK cell development in vitro in the absence of IL-15 signaling (Gascoyne et al. 2009; M. Yang et al. 2015). IL-15 is thought to drive Nfil3 expression through the kinase PDK1 and its downstream target mTOR (M. Yang et al. 2015) and mice with an NK cell specific deletion of mTOR have a block in maturation of bone marrow NK cells and a severe lack of NK cells in peripheral organs (Marçais et al. 2014). However, several recent studies have shown that certain tissue resident NK cells may be Nfil3-independent (D K Sojka et al. 2014; Cortez et al. 2014; Seillet, Huntington, et al. 2014).

B cells, T cells, and most ILCs require IL-7 for their development, whereas NK cells (von Freeden-Jeffry et al. 1995; Satoh-Takayama et al. 2010; Vonarbourg et al. 2010) and type 1 ILCs (Klose et al. 2014) do not. The first step towards an NK cell fate coincides with gain of CD122 and loss of IL-7Rα, reflecting a shift from IL-7 to IL-15 dependence. IL-15 signals through the transcription factor STAT5, and thus mice lacking Stat5b are deficient in NK cells (Imada et al. 1998). Deleting Stat5a/b in NKP also results in complete lack of NK cells (Eckelhart et al. 2011). Similarly, the transcription factor Runx3 and its binding partner Cbfβ can promote CD122 expression, and NKP deficient in these factors fail to produce peripheral NK cells in fetal liver chimeras (Guo et al. 2008). An NKp46^{+} cell-specific deletion of Runx3 also results in a lack of peripheral NK cells (Ebihara et al. 2015). T-bet and Eomes have also been shown to cooperate to promote expression of CD122 and mice lacking both these transcription factors are deficient in NK cells as well as memory CD8^{+} T cells (Intlekofer et al. 2005).

PU.1, a member of the Ets family of transcription factors (reviewed in (Hollenhorst et al. 2011)), is important in the development of T and B cells, monocytes, dendritic cells, and granulocytes (reviewed in (Carotta et al. 2010)). Although PU.1-deficient fetal liver cells are able to generate NK cells, chimeric mice have reduced numbers of NKP and immature NK cells (Colucci et al. 2001). PU.1-deficient NK cells also have increased expression of another Ets family factor, Ets-1, a finding that has led to suggestions that Ets-1 can compensate for lack of PU.1 in driving the NK cell lineage (Colucci et al. 2001). Although Ets-1 is

likely expressed prior to the NKP stage, it appears to be required at a later point in development than PU.1. Peripheral NK cells are severely decreased in Ets-1-deficient mice (Barton et al. 1998) due to an arrest at the NKP stage (Ramirez et al. 2012). Furthermore, residual NK cells in Ets-1-deficient mice were refractory when stimulated through activating receptors but hyperresponsive to proinflammatory cytokines, suggesting chronic basal stimulation (Ramirez et al. 2012). Ets-1 was shown to promote other transcription factors critical in NK cell development, including Id2 and T-bet (Ramirez et al. 2012). Similarly, mice lacking the Ets family member Mef have decreased NK cell numbers, as well as a defect in IFN-γ secretion and perforin expression (Lacorazza et al. 2002).

3. Transcription Factors Governing NK Cell Maturation[1]

In addition to the early role for Id family transcription factors in suppressing the adaptive lymphocyte fate while promoting innate lymphocyte development, these factors are also important later in the development of NK cells. Id2-deficient mice have a cell-intrinsic lack of peripheral NK cells (Yokota et al. 1999) that was found to be due to an arrest at the iNK stage (Boos et al. 2007), indicating that Id2 is important in the transition from immature to mature NK cell. Both Id2 and Id3 are expressed in NKP, and Id2 continues to be expressed in NK cells through the mature NK cell stage. In addition, both Id2 and Id3 can promote NK cell development in culture (Heemskerk et al. 1997; Schotte et al. 2010) and Id2 is thought to be downstream of Nfil3, as ectopic Id2 expression can rescue NK cell development in Nfil3 deficient progenitors (Gascoyne et al. 2009; Male et al. 2014).

The T-box family of transcription factors is critical in several aspects of NK cell development and maturation. One family member, T-bet, is thought to regulate expression of S1P5, a receptor required for NK cell egress out of lymph nodes and bone marrow (Jenne et al. 2009). T-bet-deficient mice lack mature NK cells in the bone marrow and periphery, exhibiting an arrest at the iNK stage during development (Townsend et al. 2004). A more recent study suggests that T-bet stabilizes an immature ($TRAIL^{+}DX5^{-}$) NK cell state and that loss of T-bet results in higher expression of Eomes, another T-box transcription factor (Gordon et al. 2012). Eomes is required for transition to the mature ($DX5^{+}$) NK cell stage and acquisition of Ly49 receptors (Gordon et al. 2012). However, there is some question as to whether this $TRAIL^{+}$ population truly consists of immature NK cells or whether it represents a distinct lineage of ILC1 (reviewed in (Dorothy K Sojka et al. 2014; O'Sullivan, Sun, et al. 2015)). Loss of T-bet also results in reduced expression of another transcription factor, Blimp1, which is similarly required for progression from the iNK stage (Kallies et al. 2011). The transcription factor Gata3 is thought to promote T-bet expression, as Gata3-deficient NK cells have reduced T-bet expression and are immature in phenotype similar to T-bet-deficient NK cells (Samson et al. 2003). Conversely, the forkhead box family transcription factors Foxo1 and Foxo3 suppress NK cell maturation by repressing T-bet (Deng et al. 2015). Foxo3-deficient mice have normal numbers of NK cells but an increase in $KLRG1^{+}$ NK cells, suggesting a role for Foxo3 in suppressing terminal maturation of NK cells (Huntington et al. 2007).

The transcription factor Tox is required for transition from the iNK to mNK stage, as Tox-deficient mice have a severe defect in mature NK cells but no defect in immature NK cells or NKP (Aliahmad et al. 2010). Tox is believed to be downstream of Nfil3, as transduction of Nfil3-deficient bone marrow with Tox-expressing retroviruses was able to rescue NK cell and ILC development in recipient mice (X. Yu et al. 2014). Aiolos, an Ikaros family member, is also required for terminal maturation of NK cells; Aiolos-deficient mice have an accumulation of $CD27^{+}CD11b^{-}$ and $CD27^{+}CD11b^{+}$ NK cells but a loss of the most mature $CD27^{-}CD11b^{+}$ NK cell subset (Holmes et al. 2014). Irf2 is similarly required for promoting mature NK cells, as Irf2-deficient mice maintain normal numbers of immature NK cells in the bone marrow but lack the most mature $CD11b^{+}$ subset and circulating NK cells (Lohoff et al. 2000; Taki et al. 2005). Runx3, important in promoting CD122 expression (Guo et al. 2008), has likewise been shown to be important late in NK cell development, promoting the expression of Ly49 receptors, CD11b, and CD43 (Ohno et al. 2008).

4. Regulation of Effector NK Cell Responses and Memory Formation[1]

The STAT family of transcription factors contains members that are phosphorylated downstream of pro-inflammatory cytokine receptors and form homo- or hetero-dimers that translocate to the nucleus to induce gene transcription (reviewed in (O'Shea et al. 2015)). During viral infection, type I IFNs and downstream STAT1 have been shown to enhance NK cell cytotoxicity (Figure 7) (Orange and Biron 1996; Nguyen et al. 2002), and shield activated NK cells from cell death via an NKG2D-dependent fratricide mechanism (Madera et

al. 2016). IL-12 and downstream STAT4 are required for NK cell production of IFN-γ (Orange and Biron 1996; Nguyen et al. 2002; Thierfelder et al. 1996), and NK cell expansion and memory generation after mouse cytomegalovirus (MCMV) infection (Sun et al. 2012). IL-33, IL-18, and MyD88 are also important for optimal expansion during viral infection, but are not required for memory cell formation (Madera and Sun 2015; Nabekura et al. 2015). In addition, IL-12, IL-18, and type I IFNs together drive expression of the transcription factor Zbtb32, which promotes NK cell proliferation after MCMV infection by antagonizing Blimp-1 (Beaulieu et al. 2014). The aryl hydrocarbon receptor (AhR) is another nuclear factor required for optimal cytotoxicity of NK cells (Shin et al. 2013). Similarly, NK cells deficient in either of the transcription factors C/EBP or MITF have reduced cytotoxicity and IFN-γ secretion (Kaisho et al. 1999; Seaman et al. 1979; Ito et al. 2001; Kataoka et al. 2005). MITF may regulate cytotoxicity through interactions with MEF and PU.1 at the perforin promoter (Lacorazza et al. 2002; Hesslein and Lanier 2011).

Control of the apoptosis pathway is thought to be involved in regulating NK cell memory formation, as the anti-apoptotic molecule Bcl-2 is downregulated in NK cells following MCMV infection (Beaulieu et al. 2014; Min-Oo et al. 2014). The pro-apoptotic factor Bim controls the formation of memory NK cells, with Bim-deficient NK cells failing to contract normally following MCMV infection and displaying lower levels of memory-associated cell surface markers (Min-Oo et al. 2014). A recent study found that NK cells accumulate damaged mitochondria after MCMV infection, and that a small subset that cleared these mitochondria by

autophagy preferentially survived to form memory NK cells, a process dependent on BNIP3 and BNIP3L (O'Sullivan, Johnson, et al. 2015). Additional factors that may be specifically required for memory formation remain to be elucidated.

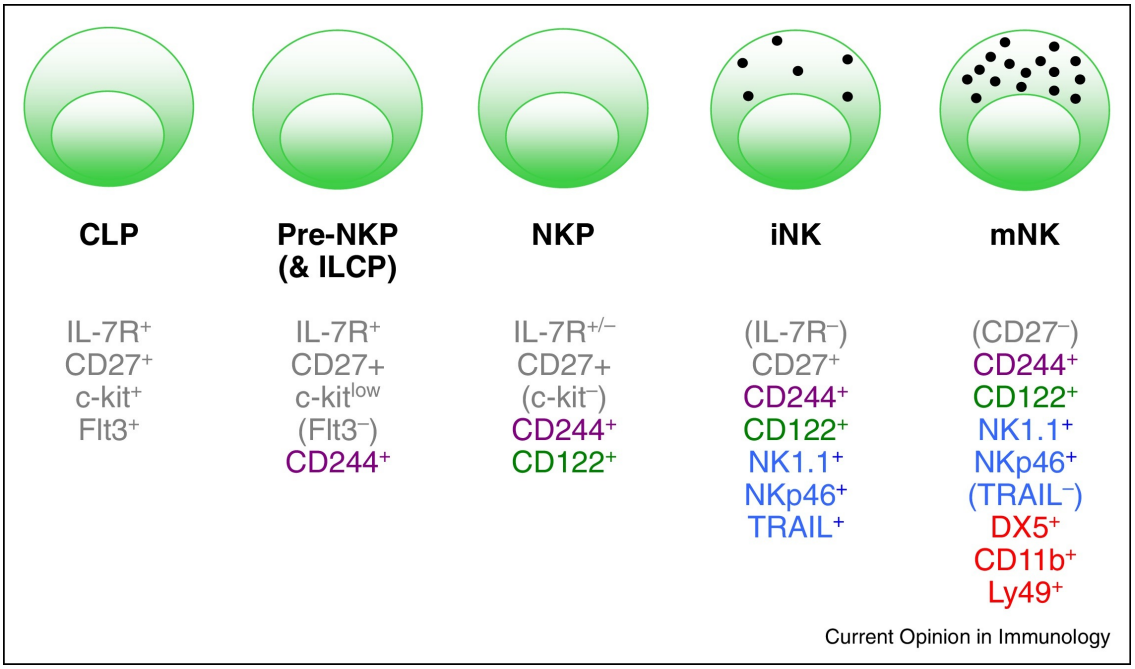

FIGURE 5 | STAGES OF NATURAL KILLER CELL DEVELOPMENT

NK cells are derived from the CLP, which differentiates into a heterogeneous pre-NKP/ILCP population distinguished from the NKP by its expression of IL-7R and lack of CD122 expression. From the NKP, cells begin to express NK cell markers NK1.1 and NKp46, and as they further mature they acquire expression of DX5 (CD49b) and CD11b while losing expression of CD27. As NK cells mature they also gain functional competence, expressing lytic molecules and cytokines such as IFN-γ. Cell surface proteins are color coded by the stage in which they are first expressed. Loss of a specific cell surface marker after a given stage is indicated by parentheses in the stage immediately following.

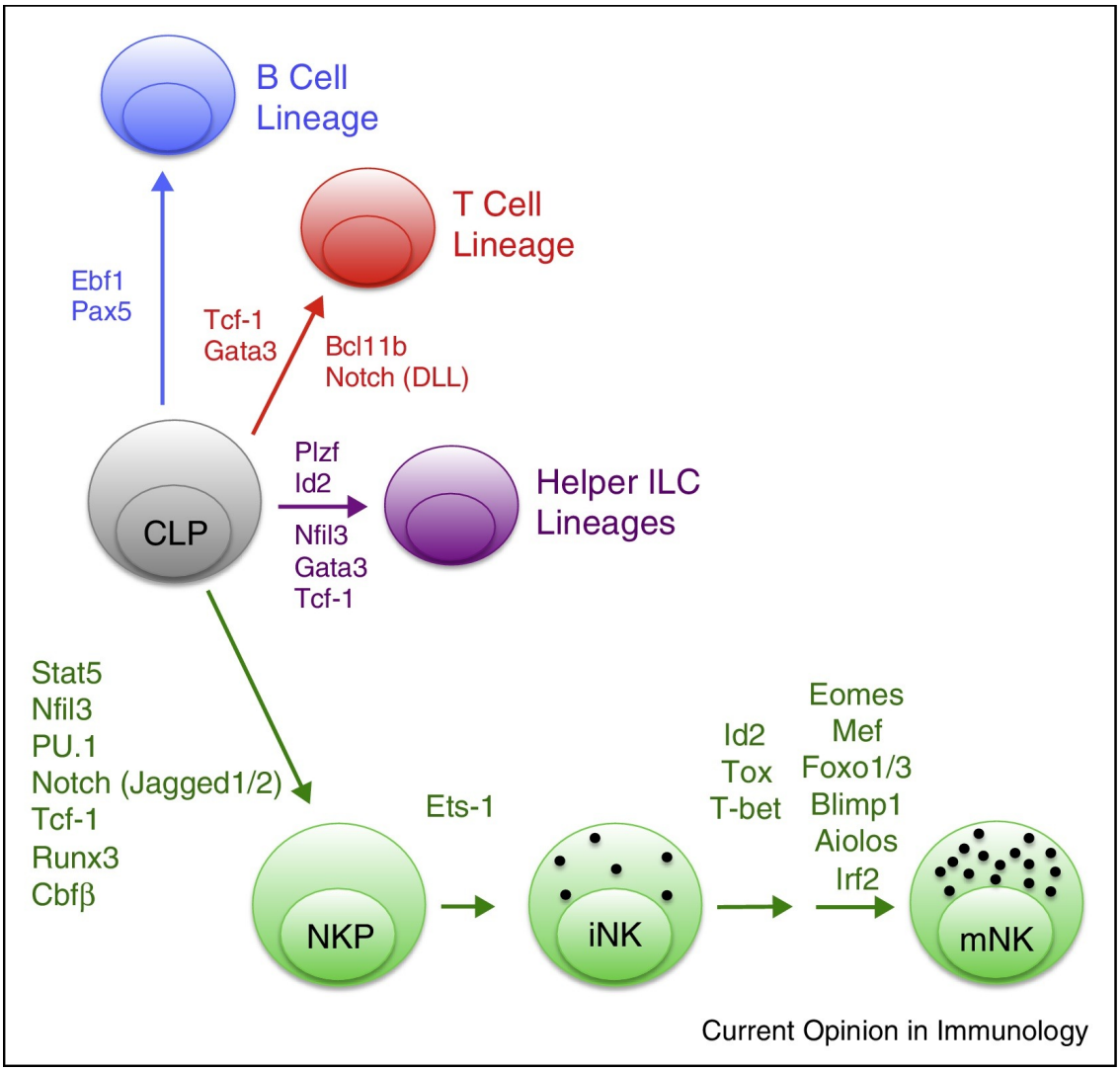

FIGURE 6 | TRANSCRIPTIONAL CONTROL OF THE NK CELL LINEAGE

A complex network of transcription factors governs the decision to adopt an innate or adaptive lymphocyte fate from the CLP. A simplified list of factors promoting the helper ILC, T cell, and B cell fates is shown. From the CLP, the indicated transcription factors drive cells to become NKP, immature NK cells (iNK) and mature NK cells (mNK). Factors promoting transition from the iNK to mNK stage are listed in two columns based on whether they are thought to be important earlier (left) or later (right) in NK cell maturation. Transcription factors are placed based upon where a defect or development arrest is seen in the knockout mouse when possible. Expression of the factors may occur before the indicated stage.

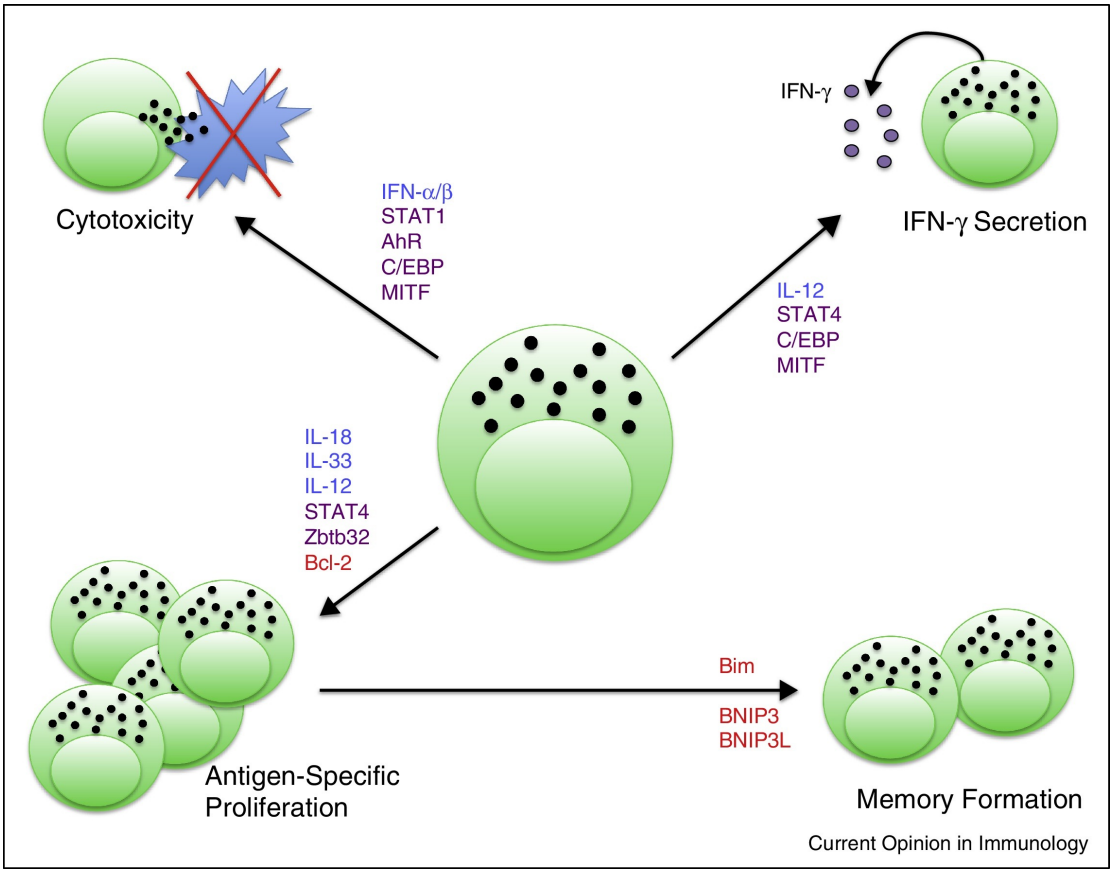

FIGURE 7 | REGULATION OF NK CELL FUNCTION AND MEMORY

Activated NK cells can secrete lytic molecules and IFN-γ. They can also proliferate in response to specific antigens and contract to form long-lived 'memory' NK cells. Different cytokines (blue), transcription factors (purple), and other molecules (red) govern each of these distinct NK cell functions.

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III. ILCs in Disease and Homeostasis

1. NK cells and ILC1

NK cells were first identified in 1975 as innate cytotoxic lymphocytes with the ability to rapidly kill tumor cells (Kiessling, Klein, Pross, et al. 1975; Kiessling, Klein and Wigzell 1975; Herberman et al. 1975). One way in which NK cells can recognize and kill tumor cells is by recognizing tumor cell downregulation of major histocompatibility complex I (MHC-I). The presentation of MHC-I on a cell surface is an inhibitory signal to NK cells, which renders stressed or infected cells that have downregulated MHC-I expression susceptible to killing by NK cells. This concept is known as the "missing self" hypothesis (Ljunggren and Kärre 1985; Kärre et al. 1986; Ljunggren and Kärre 1990). NK cells also play a critical role in host protection against infectious disease and particularly against herpesviruses including human cytomegalovirus (HCMV) (Orange 2006; Biron et al. 1989; Etzioni et al. 2005).

Mouse cytomegalovirus (MCMV) provides an excellent model to study NK cell-mediated protection from viral infection. Mice with NK cell deficiencies are extremely susceptible to MCMV infection (Shellam et al. 1981; Bancroft et al. 1981). Depletion of NK cells with antibodies against NK cell receptors results in increased viral burden and mortality in both normal and immunodeficient (lacking T and B cells) mice (Shanley 1990; Welsh et al. 1990; Bukowski et al. 1984; Welsh et al. 1994; Bukowski et al. 1985; Welsh et al. 1991). MCMV also allows the study of antigen-specific NK cell responses and memory formation, as it has been shown that in C57BL/6 mice NK cells expressing the Ly49H activating

receptor respond specifically to the MCMV-encoded m157 glycoprotein expressed by infected cells (Figure 8) (Arase et al. 2002; Dokun et al. 2001; Brown et al. 2001; Daniels et al. 2001; Scalzo et al. 1990; Smith et al. 2002). Though NK cells were classically thought to be strictly part of innate immunity, there have now been many studies showing NK cells can form long-lived memory-like cells (Sun et al. 2009; Paust and von Andrian 2011; Vivier et al. 2011; O'Leary et al. 2006). Formation of memory cells can be induced by cytokine exposure or in response to specific antigens (Min-Oo et al. 2013). In the case of MCMV infection, Ly49H$^+$ NK cells preferentially expand and later contract to form MCMV-specific memory NK cells that possess a unique transcriptional signature and are more adept at protecting from a secondary infection compared to naïve NK cells (Figure 8) (Sun et al. 2009; O'Sullivan, Sun, et al. 2015).

Not much is yet known about the contribution of non-NK cell ILC1 to immunity. ILC1 have been shown to accumulate in the intestines of Crohn's disease patients and contribute to pathology in a mouse model of colitis, indicating that ILC1 derived IFN-γ may contribute to gut inflammation (Figure 9) (Bernink et al. 2013; Fuchs et al. 2013). A recent study has also identified a novel ILC1-like cell that contributes to tumor immunosurveillance in a mouse mammary tumor model (Dadi et al. 2016).

2. ILC2

ILC2s were first identified as a cell population important for host protection against helminths due to their rapid production of type 2 cytokines (Fallon et al. 2006). ILC2s are induced to produce type 2 cytokines by IL-25 and IL-33,

produced by various cell types including alveolar macrophages, mast cells, eosinophils, basophils, and epithelial cells (Hwang and McKenzie 2013). ILC2s and their production of IL-13 are critical for expulsion of the helminth *Nippostrongylus brasilienis* (Figure 9) (Moro et al. 2010). Mice lacking ILC2s or with an ILC2 specific deletion of IL-13 are unable to clear *N. brasilienis* infection (Neill et al. 2010). Suprisingly, T_h2 derived type 2 cytokines are not necessary for clearance of *N. brasilienis* (Voehringer et al. 2006), but the presence of T_h2 cells contributes to immunity, potentially by supporting ILC2 maintenance (Neill et al. 2010). Conversely, ILC2 expression of MHC-II is necessary for optimal T_h2 responses against *N. brasilienis* (Oliphant et al. 2014), suggesting that ILC2-T_h2 crosstalk supports both cell types in anti-helminth immunity. ILC2s also contribute to wound healing and tissue repair by producing the growth factor amphiregulin, which induces proliferation of epithelial cells (Figure 9) (Monticelli et al. 2011; Enomoto et al. 2009). Conversely, ILC2s can also be pathogenic and have been shown to contribute to airway hyperreactivity during influenza infection or allergic inflammation (Figure 9) (Chang et al. 2011; Kim et al. 2012; Barlow et al. 2012; Klein Wolterink et al. 2012; Bartemes et al. 2012; Halim, Krauss, et al. 2012).

3. ILC3 and LTi Cells

LTi cells are critical for formation of lymph nodes and Peyer's patches (Eberl et al. 2004; Kelly and Scollay 1992; Eberl and Littman 2003; Mebius et al. 1997). In early lymphoid organ development they interact with stromal organizer cells to promote expression of adhesion molecules such as vascular cell

adhesion molecule 1 (VCAM1) and chemokines such as chemokine ligand 19 (CCL19) and CCL21 (Figure 9) (van de Pavert and Mebius 2010). Postnatally, LTi cells are also thought to be important for formation of isolated lymphoid follicles and for repair of lymph nodes damaged by infection (Scandella et al. 2008; Hamada et al. 2002; Bouskra et al. 2008; Hwang and McKenzie 2013).

ILC3-produced IL-22 is thought to be critical for host defense against intestinal bacterial infections such as *Citrobacter rodentium* and vancomycin-resistant *Enterococcus faecium* (VRE) (Zheng et al. 2008; Cella et al. 2009; Satoh-Takayama et al. 2008; Abt et al. 2016), as well as for host resistance to colitis (Figure 9) (Cox et al. 2012). Furthermore, IL-22 produced by ILCs can protect mice from graft-versus-host disease (GVHD)-associated or chemotherapy-induced intestinal damage by protecting intestinal stem cells and inducing them to promote epithelial regeneration (Hanash et al. 2012; Lindemans et al. 2015; Aparicio-Domingo et al. 2015; Munneke et al. 2014). ILC3s can also mediate immune responses independently of their ability to secrete cytokines. A recent study has shown that they express major histocompatibility complex II (MHC-II) and can therefore present antigen to $CD4^+$ T cells (Figure 9) (Hepworth et al. 2013). This interaction is thought to limit $CD4^+$ T cell responses against commensal microbiota and mice lacking MHC-II on ILC3 exhibit abnormal intestinal inflammation (Hepworth et al. 2013). Conversely, ILC3s can be pathogenic, as they are required to induce colitis in a bacteria-driven colitis model as well as the anti-CD40 colitis model (Buonocore et al. 2010) and are

thought to contribute to epidermal thickening in psoriasis patients (Figure 9) (Teunissen et al. 2014; Villanova et al. 2014).

IV. Summary

ILCs are an important subset of lymphocyte that is critical for early defenses against bacteria, viruses, parasites, and tumors. The development and function of these cells are exquisitely controlled by a vast array of transcription factors and cytokines. Here, we describe the identification of the transcription factor Nfil3 as an essential component in the developmental pathway of all ILC subsets and the subsequent disease susceptibility of *Nfil3*$^{-/-}$ mice. We also describe the dispensability of cell-intrinsic TGF-β signaling in the development of conventional peripheral NK cells and the role of TGF-β in the generation and maintenance of virus-specific memory NK cells.

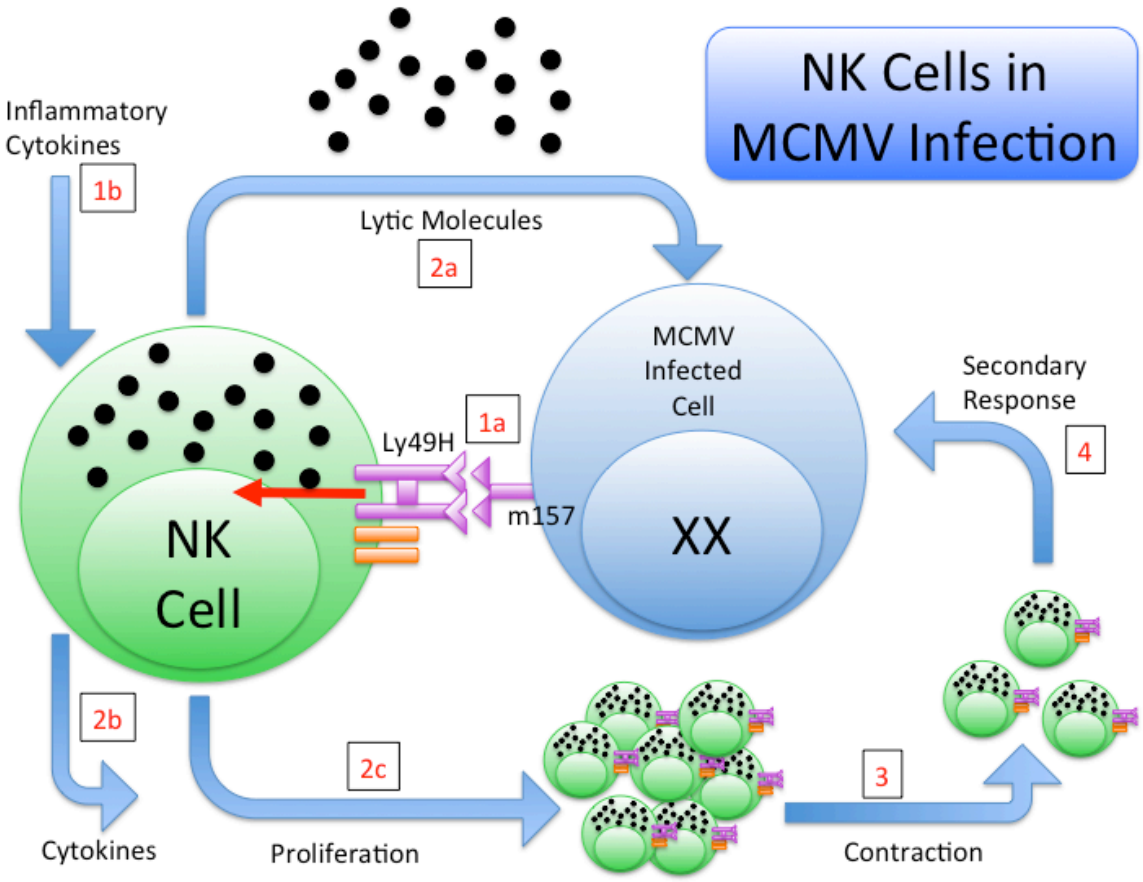

FIGURE 8 | NK CELL RESPONSES TO MCMV INFECTION

MCMV infected cells express the virally-encoded m157 glycoprotein on their cell surface, which is recognized by the Ly49H activating receptor on NK cells (1a). Receptor engagement in combination with pro-inflammatory cytokines (1b) induce the NK cell to release lytic molecules (2a) to kill the target cell, as well as to release pro-inflammatory cytokines such as IFN-γ (2b). Ly49H^{+} NK cells will also clonally proliferate in response to these activating stimuli (2c). These MCMV-specific NK cells will later contract (3) to form a long-lived memory pool that can be reactivated upon secondary infection (4).

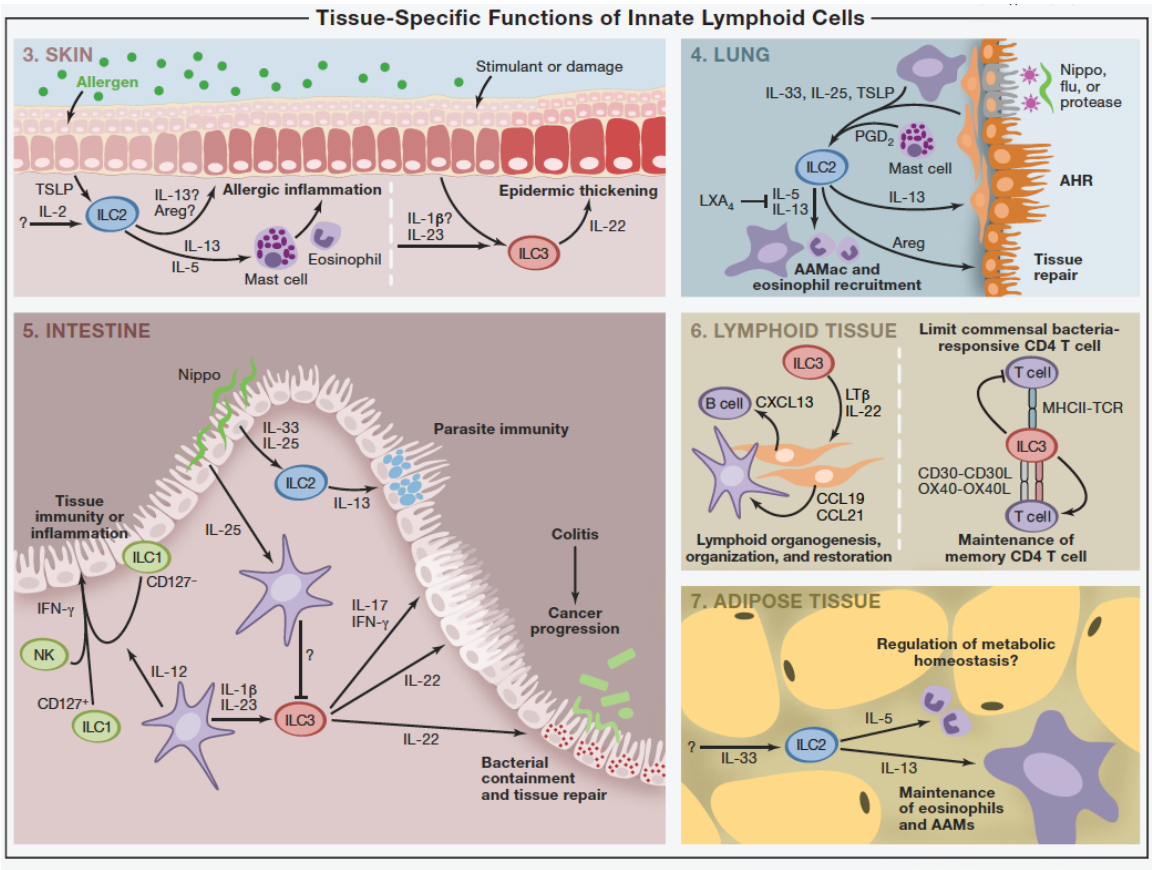

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FIGURE 9 | INNATE LYMPHOID CELL FUNCTION

ILCs perform many different functions in immunity. In the skin (3) ILC2s contribute to allergic inflammation while ILC3s can promote epidermal thickening. In the lung (4) ILC2s contribute to airway hyperreactivity as well as immunity to parasites. They can also aid tissue repair by producing amphiregulin. In the intestine (5) ILC1s contribute to inflammation, while ILC2s aid in parasite immunity and ILC3s can mediate colitis or tissue repair. In lymphoid tissue (6) LTi cells promote lymphoid organogenesis by releasing cytokines and promoting chemokine expression. ILC3 can also limit $CD4^+$ T cell responses by expression of MHC-II. In the adipose tissue (7), ILC2 recruit eosinophils.

Chapter 2: Nfil3 is Crucial for the Development of all Innate Lymphoid Cells

I. Introduction

With their importance in antimicrobial and autoimmune functions as well as in tissue homeostasis and wound healing, there is currently great interest in understanding exactly how ILCs develop and function. As previously described, ILCs require IL-7 and the IL-2 receptor common γ chain for their development, but do not undergo antigen receptor rearrangement (Spits and Di Santo 2011; Spits and Cupedo 2012). Transcription factors required to promote differentiation of different ILC lineages were outlined in Chapter 1. We were one of the first groups to identify the requirement for nuclear factor IL-3 regulated (Nfil3, also called E4BP4) in the development of all ILC lineages. This discovery has given us great insight into the early stages of ILC development, and *Nfil3*$^{-/-}$ mice have proved to be a useful tool to study the role of ILCs in infection and cancer.

1. The Transcription Factor Nfil3/E4BP4

Nfil3 is a member of the basic leucine zipper (bZIP) family of transcription factors, which is comprised of five main classes: CREB/ATF, AP1, C/EBP, NF-E2, and PAR (Cowell 2002). Nfil3 is most closely related to the PAR class of bZIP factors, which is defined by factors containing a large basic region, highly similar DNA-binding sequences, and a proline and acid residue rich (PAR) region. Nfil3 contains the basic region and has a PAR-like DNA binding sequence, but lacks the PAR domain (Cowell 2002). Although PAR factors are

typically activators, Nfil3 has been reported to be a transcriptional repressor in some contexts (Cowell et al. 1992; Cowell and Hurst 1994; Kobayashi et al. 2011). However, Nfil3 has also been reported to act as a transactivator in T cells, inducing the transcription of IL-3 (Zhang et al. 1995). The activity of Nfil3 as an activator or repressor is likely determined by the cell type-specific availability of cofactors (Cowell 2002).

2. Nfil3 in the Immune System

Nfil3 controls a wide range of functions in many immune cell types (Figure 10). It has been shown to induce transcription of IL-3 in T cells (Zhang et al. 1995) and promotes production of IL-10 and IL-13 by Th1 cells (Motomura et al. 2011), but suppresses type 2 cytokine production by T_h2 cells (Kashiwada, Cassel, et al. 2011). It promotes survival of pro-B cells and is also required for class-switching to IgE (Ikushima et al. 1997; Kashiwada et al. 2010). It suppresses transcription of IL-12 p40 in macrophages (Kobayashi et al. 2011) and is required for the development of $CD8^+$ dendritic cells, possibly upstream of BATF3 (Kashiwada, Pham, et al. 2011). Furthermore, it has been shown to suppress T_h17 development in a manner linked to the circadian clock (Yu et al. 2013). However, the most striking phenotype in *Nfil3*$^{-/-}$ mice is the near complete absence of NK cells and ILC1 (Firth et al. 2013; Fuchs et al. 2013; Gascoyne et al. 2009; Kamizono et al. 2009; Kashiwada et al. 2010). The role of Nfil3 in other ILC lineages has only recently been elucidated.

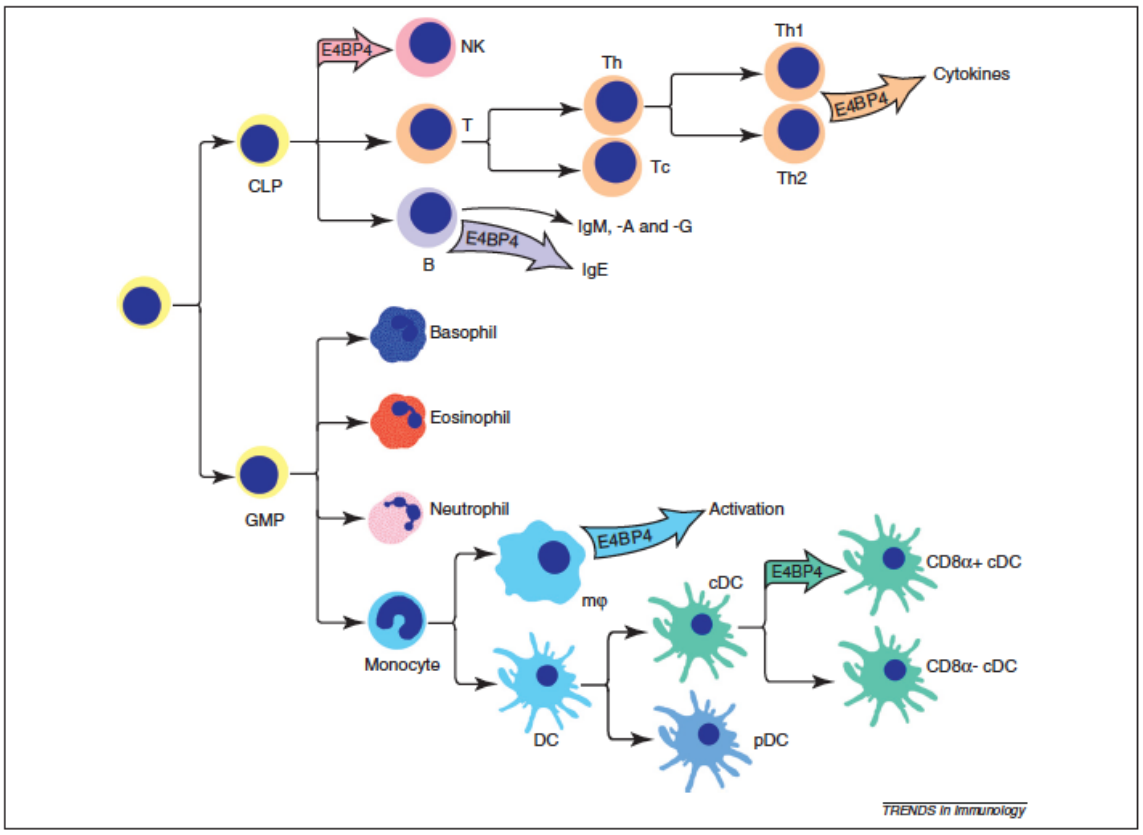

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FIGURE 10 | NFIL3 IN IMMUNE CELLS

Nfil3 (E4BP4) has many roles in immune cells. It is required for the development of NK cells and $CD8^{+}$ dendritic cells. It is also required for B cell class switching to IgE and promotes survival of pro-B cells. It suppresses IL-12 production by macrophages and affects cytokine production in T cells.

II. Results

1. Intestinal Group 3 ILCs are Severely Reduced in Nfil3-deficient Mice[2]

Consistent with previously reported findings (Firth et al. 2013; Fuchs et al. 2013; Gascoyne et al. 2009; Kamizono et al. 2009; Kashiwada et al. 2010), we found a cell-intrinsic requirement for Nfil3 in NK cells and ILC1 in multiple tissues, including small intestine (SI), mesenteric lymph node (mLN), and spleen (Figure 11). Given that NK cells and ILC1s are found at extremely reduced frequency in *Nfil3*$^{-/-}$ mice at steady state, we investigated whether Nfil3 was also required for development or homeostasis of other innate lymphocyte populations. Because ILCs (identified as lineage-negative cells that co-express CD45, IL-7Rα (CD127), and Thy 1 (CD90)) are found in relatively high abundance at gut mucosal sites (Sonnenberg et al. 2013; Spits et al. 2013; Walker et al. 2013), we analyzed these innate lymphocytes (Figure 12A) in the lamina propria of small intestine (SI) and large intestine (LI), and in Peyer's patches (PP) of WT and *Nfil3*$^{-/-}$ mice. In contrast to WT mice, *Nfil3*$^{-/-}$ mice contained severely diminished ILC3 numbers in all intestinal sites examined (Figure 12B). The defect in ILC3 numbers in the gut was also observed in mesenteric lymph nodes and spleen of *Nfil3*$^{-/-}$ mice (Figure 12B), suggesting that the defect was not due to an inability to properly home to mucosal sites. Furthermore, the few residual intestinal ILC3s identified phenotypically from *Nfil3*$^{-/-}$ mice were functionally impaired in their ability to produce IL-22 when stimulated ex vivo with IL-23 (Figure 12C). Within the RORγt^{+} ILC3 population, intestinal CD4-expressing LTi cells from *Nfil3*$^{-/-}$ mice were also dramatically reduced compared to WT mice (Figure 12D), as were

both NKp46^{-} and NKp46^{+} ILC3s (Figure 12E), demonstrating the critical role of Nfil3 for the development of all type 3 ILCs.

2. Cell-intrinsic Requirement for Nfil3 in ILC3 Development[2]

To rule out the possibility that ILC-extrinsic factors in *Nfil3*$^{-/-}$ mice may underlie the observed ILC3 defects, we generated mixed bone marrow chimeric mice where lethally irradiated, congenically-distinct recipient mice (CD45.1) received a 1:1 mixture of bone marrow from WT (CD45.1x2) and *Nfil3*$^{-/-}$ (CD45.2) mice. We analyzed the mice 8-12 weeks following bone marrow transplantation (BMT), as we have previously observed development of donor ILC3s in recipient intestines at this time post-BMT (Hanash et al. 2012). Although there were no substantial differences in myeloid, T, or B cell chimerism (Figure 13), intestinal ILC3s from the WT donor population greatly outnumbered the ILC3s from the *Nfil3*$^{-/-}$ donor population (Figure 14A). In the chimeric mice, ILC3 development from *Nfil3*$^{-/-}$ donor marrow was impaired in multiple compartments, including SI, LI, and PP, compared to the WT donor population (Figure 14B). Furthermore, upon ex vivo stimulation of total ILC3s with IL-23, the IL-22-producing cells were overwhelmingly found within the WT population (Figure 14C). Because the mixed chimera setting possesses both WT stromal and hematopoietic elements, our findings imply that Nfil3 acts in a cell-intrinsic manner to drive ILC3 development and/or homeostasis.

3. Nfil3 is Essential for Resistance Against Intestinal Pathogens[2]

ILC3s have been shown to be critical for host protection against the murine enteric pathogen *Citrobacter rodentium*, as mice lacking ILC3s or

depleted of ILCs become susceptible to bacterial dissemination and mortality (Cella et al. 2009; Qiu et al. 2012; Satoh-Takayama et al. 2008; Sonnenberg et al. 2012; Sonnenberg et al. 2011). Given the defective ILC3 numbers in *Nfil3*$^{-/-}$ mice compared to WT mice, we next investigated whether *Nfil3*$^{-/-}$ mice were more susceptible to oral challenge with *C. rodentium*. In our studies, WT and *Nfil3*$^{-/-}$ mice, along with *Nfil3*$^{+/-}$ heterozygous control mice containing intact ILC3 development, were co-housed for a minimum of 2-3 weeks before infection to ensure normalization of mouse commensal microbial communities (Elinav et al. 2011; Ubeda et al. 2012). Following oral *C. rodentium* infection, all three experimental cohorts were assessed for disease status and bacterial titers (Figure 15A). Within 4 days post-infection (PI), *Nfil3*$^{-/-}$ mice began to lose body weight at a greater rate than WT mice or *Nfil3*$^{+/-}$ littermates (Figure 15B), despite comparable C. rodentium titers in all experimental groups early after infection (Figure 16). The *Nfil3*$^{-/-}$ mice showed significantly greater weight loss at day 7 and 11 PI, whereas WT and *Nfil3*$^{+/-}$ mice maintained body weight (Figure 15B). All groups were sacrificed at day 11 PI and *Nfil3*$^{-/-}$ mice had higher bacterial titers within cecal contents (Figure 15C) with some showing bacterial dissemination to the liver (Figure 16), compared with control groups. Consistent with *C. rodentium*-induced colitis, infected *Nfil3*$^{-/-}$ mice had shorter colons relative to WT and *Nfil3*$^{+/-}$ mice (Figure 15D), even though we have not observed shorter colons in uninfected *Nfil3*$^{-/-}$ mice (Figure 17). Finally, WT but not *Nfil3*$^{-/-}$ ILC3s dominated the total intestinal ILC3 population in chimeric mice infected with *C. rodentium* (Figure 15E), suggesting that inflammation generated during infection is unable

to expand or recruit gut ILC3s lacking Nfil3. The inability of ILC3s to undergo prolific expansion was confirmed by the lack of BrdU incorporation in mice infected with either *C. rodentium* or MCMV (Figure 18), the latter of which was previously shown to drive Ly49H^{+} NK cells to expand in *Nfil3*$^{-/-}$ mice (Firth et al. 2013).

Next, we tested susceptibility of *Nfil3*$^{-/-}$ mice against pathogenic bacteria using a clinically relevant model of intestinal *Clostridium difficile* infection. *C. difficile* is an opportunistic gram-positive bacterium that can cause severe colitis and diarrhea when the normal microbiota is disrupted following antibiotic treatment (Rupnik et al. 2009) and the incidence of infection in hospital settings is increasing, especially among BMT patients (Kelly and LaMont 2008). As with the *C. rodentium* model, experimental mice were first co-housed for 2-3 weeks; mice were then treated with an antibiotic regimen (diagrammed in Figure 15F) previously shown to disrupt the intestinal microbiota and induce susceptibility to *C. difficile* spores and colitis (Buffie et al. 2012). Antibiotic-treated *Nfil3*$^{-/-}$ mice orally challenged with a pathogenic strain of *C. difficile* demonstrated extreme weight loss within 48-72 hours PI, in contrast to WT and *Nfil3*$^{+/-}$ heterozygous mice (Figure 15G). Within 3 days PI, all of the *Nfil3*$^{-/-}$ mice succumbed to *C. difficile* infection whereas control groups recovered from initial weight loss (Figure 15H). Together with the *C. rodentium* studies, infection with *C. difficile* demonstrates that the transcription factor Nfil3 contributes to host protection against multiple intestinal bacterial pathogens.

4. Development of the ILC Precursor Depends on Nfil3[2]

To better understand at which developmental stage Nfil3 is required for generation of mature ILC3s, we analyzed the expression level of Nfil3 mRNA in the earliest progenitor cells by microarray, and in ILC precursors and mature ILC3s by qRT-PCR. We found that Nfil3 expression increases as the hematopoietic stem cell (HSC) differentiates into the multi-potent progenitor (MPP) and then the Id2-expressing common lymphoid progenitor (CLP) (Figure 19A). Indeed, these data are consistent with recent findings demonstrating Nfil3 expression as early as the CLP stage (Male et al. 2014). From the CLP to ILC precursor (ILCP) to mature ILC3 stage, Nfil3 expression continues to increase, with highest levels of Nfil3 in gut ILC3 (Figure 19A). Thus, we analyzed CLP (Lin$^-$ CD45$^+$ CD27$^+$ CD127$^+$ c-Kit$^+$) and ILCP (Lin$^-$ CD45$^+$ CD27$^+$ CD127$^+$ c-Kit$^+$ CD135$^-$ α4β7$^+$) populations in the bone marrow of WT and *Nfil3*$^{-/-}$ mice (Figure 19B), using surface markers previously described to delineate these precursors (Fathman et al. 2011; Cherrier et al. 2012; Possot et al. 2011; Sawa et al. 2010; Serafini et al. 2014; Walker et al. 2013). Whereas CLP numbers in the bone marrow were comparable between WT and *Nfil3*$^{-/-}$ mice, ILCP numbers were strikingly reduced in *Nfil3*$^{-/-}$ mice (Figure 19C), suggesting that Nfil3 is required for the transition from the CLP stage to the ILCP stage. Thus, it is possible that Nfil3 is expressed earlier than and may regulate the expression of RORγt and Gata3, neither of which is expressed in CLP, although ILCP expressed Gata3 (Figure 19D), consistent with recent findings (Serafini et al. 2014).

5. Nfil3-independent Maintenance of Mature NKp46^{+} ILC3s[2]

Expression of Nfil3 in mature ILC3s is greater than in conventional NK cells (Figure 19A) (Klose et al. 2014), even though maintenance of mature NK cells is Nfil3-independent (Firth et al. 2013). Our data and previous studies have found that a large fraction of intestinal ILC3s express the activating receptor NKp46 (Figure 12E) (Cella et al. 2009; Sanos et al. 2009; Satoh-Takayama et al. 2008; Sawa et al. 2010). To investigate whether Nfil3 is required for maintenance of a mature ILC3 population beyond the developmental stage when NKp46 is first expressed, we crossed *Nkp46*^{iCre} mice (Narni-Mancinelli et al. 2011), which express Cre-recombinase under control of the NKp46 gene, to *Nfil3*$^{fl/fl}$ mice (Motomura et al. 2011). *Nfil3*$^{fl/fl}$ *x Nkp46*^{iCre} mice contained a normal number of mature intestinal ILC3s compared to littermate control mice lacking Cre expression (Figure 19E). Similar ILC3 frequencies were also found in the spleen and mesenteric lymph nodes of *Nfil3*$^{fl/fl}$ *x Nkp46*^{iCre} mice and littermate controls (Figure 19F). Together, these data suggest that Nfil3 is required for ILC3s at a developmental stage preceding the acquisition of NKp46 expression, and that the maintenance of NKp46^{+} ILC3s is independent of Nfil3.

6. ILC2 Populations are Severely Diminished in Nfil3-deficient Mice[2]

Given the dependence of ILC3s on Nfil3, as well as the previously reported dependence of Type 1 ILCs on Nfil3 (Firth et al. 2013; Fuchs et al. 2013; Gascoyne et al. 2009; Kamizono et al. 2009; Kashiwada et al. 2010), we investigated whether type 2 ILCs are also diminished in Nfil3-deficient mice. ILC2s have been characterized as the predominant subset of ILC in healthy

lungs, and can mediate lung inflammatory responses and pulmonary immunity against pathogens (Sonnenberg et al. 2013; Spits and Cupedo 2012; Walker et al. 2013). We discovered that *Nfil3*$^{-/-}$ mice contain markedly reduced numbers of ILC2s (identified as Lineage-negative cells that co-express CD45, IL-7Rα (CD127), Thy 1 (CD90), and Gata3) (Figure 20A) in lung tissue relative to WT mice (Figure 20B). Because ILC2s have also been described to constitute a major source of T_h2 cytokines in visceral adipose tissue (VAT) (Molofsky et al. 2013; Moro et al. 2010), we investigated whether ILC2s were defective in the VAT of *Nfil3*$^{-/-}$ mice. Indeed, compared to WT mice, both ILC2 numbers and function (as measured by IL-13 secretion) were drastically diminished within VAT of *Nfil3*$^{-/-}$ mice (Figure 20C-D), demonstrating that the ILC2 defect in the absence of Nfil3 is not restricted to the lungs. A recent study showed that a consequence of ILC2 presence in the lungs is the regulation of basal eosinophil homeostasis (Nussbaum et al. 2013). When we assessed eosinophils in the lungs of *Nfil3*$^{-/-}$ mice, we found diminished numbers compared to WT mice (Figure 20E), suggesting that Nfil3 control of ILC2 development may contribute to regulating tissue eosinophil accumulation at steady state. Using 1:1 WT:*Nfil3*$^{-/-}$ mixed chimeric mice, as described earlier (Figure 14), we found that ILC2 in lung and VAT consisted of cells derived from WT marrow in significantly greater frequency than from *Nfil3*$^{-/-}$ marrow (Figure 20E-F), suggesting that like ILC3s, development of ILC2s requires Nfil3 activity via a cell-intrinsic mechanism. Altogether, these findings demonstrate that Nfil3 deficiency results in the disrupted development of ILC1, ILC2, and ILC3 subsets.

7. *Nfil3*$^{-/-}$ and WT Mice Develop Similar Levels of Colitis After DSS Treatment

Given the susceptibility of *Nfil3*$^{-/-}$ mice to intestinal infections, we next wanted to examine whether they would be more susceptible to colitis induced by dextran sulfate sodium (DSS). DSS directly damages colonic epithelial cells and results in inflammation and destruction of colonic crypts (Ni et al. 1996; Cooper et al. 1993; Okayasu et al. 1990). Clinically, mice display diarrhea, rectal bleeding, and weight loss (Okayasu et al. 1990). These features make it a useful mouse model of ulcerative colitis and inflammatory bowel disease (Chassaing et al. 2014). It is particularly suitable for our studies because adaptive immunity is not thought to play a major role in pathology (Dieleman et al. 1994). As *Nfil3*$^{-/-}$ mice had previously shown to develop spontaneous colitis (Kobayashi et al. 2014), we hypothesized that *Nfil3*$^{-/-}$ mice would be more susceptible to DSS-induced colitis. For this reason, we first titrated the DSS dose on WT mice (Figure 21), and chose a relatively low dose (1.5%) that was able to induce mild disease in WT mice with no morbidity. However, at this low dose, we found that *Nfil3*$^{-/-}$ mice lost a similar amount of weight (Figure 22B) and had a similar disease activity index (DAI) (Cooper et al. 1993) (Figure 22C) compared to their WT littermates. Similarly, with 1.8% DSS treatment we do not observe a significant difference in disease severity between *Nfil3*$^{-/-}$ and WT mice (Figure 22D-F). In addition, *Nfil3*$^{-/-}$ mice had similar colonic shortening, a measure of colitis severity (Figure 22F), compared to their littermate controls after 1.8% DSS treatment. However, at moderate doses of DSS, *Nfil3*$^{-/-}$ mice may be slightly more susceptible to

disease. With 2.0% DSS treatment, *Nfil3*$^{-/-}$ mice lose a similar amount of weight (Figure 23A) and have a similar DAI (Figure 23B), but have slightly increased morbidity (Figure 23C), though this difference was not statisitically significant. Increasing the dose to 2.5% DSS resulted in slightly more weight loss in *Nfil3*$^{-/-}$ mice compared to WT and heterozygous littermates (Figure 23D), and *Nfil3*$^{-/-}$ mice had an increased DAI after 7 days of DSS treatment (Figure 23E).

8. *Nfil3*$^{-/-}$ Mice are Less Susceptible to AOM/DSS Polyp Formation

Having seen that *Nfil3*$^{-/-}$ mice are more susceptible to intestinal infections, we further wanted to investigate whether they would be more or less susceptible to colorectal cancer. A previous study had shown that IL-22 produced by ILC3s was tumor promoting in a bacteria-driven model of colon cancer (Kirchberger et al. 2013). We therefore hypothesized that *Nfil3*$^{-/-}$ mice, lacking all ILC lineages, may be less susceptible to a model of chemically-induced colitis-driven colon cancer. In this model, mice are injected with a genotoxic carcinogen, azoxymethane (AOM) and then subjected to successive cycles of the colitis-inducing detergent dextran sulfate sodium (DSS) (Tanaka et al. 2003; De Robertis et al. 2011; Thaker et al. 2012). The combination of the genotoxic stress and inflammation drives the formation of colonic neoplasms including adenomas and adenocarcinomas (Tanaka et al. 2003). After establishing a dose where *Nfil3*$^{-/-}$ mice could tolerate DSS treatment (Figure 22) we subjected WT or Nfil3-deficient mice to the AOM/DSS model of colon cancer (Figure 24A). In this model, we found that *Nfil3*$^{-/-}$ mice had fewer large tumors (Figure 24C) and fewer

tumors overall (Figure 24D) compared to their littermate control mice, indicating that ILCs may be tumor promoting in this model.

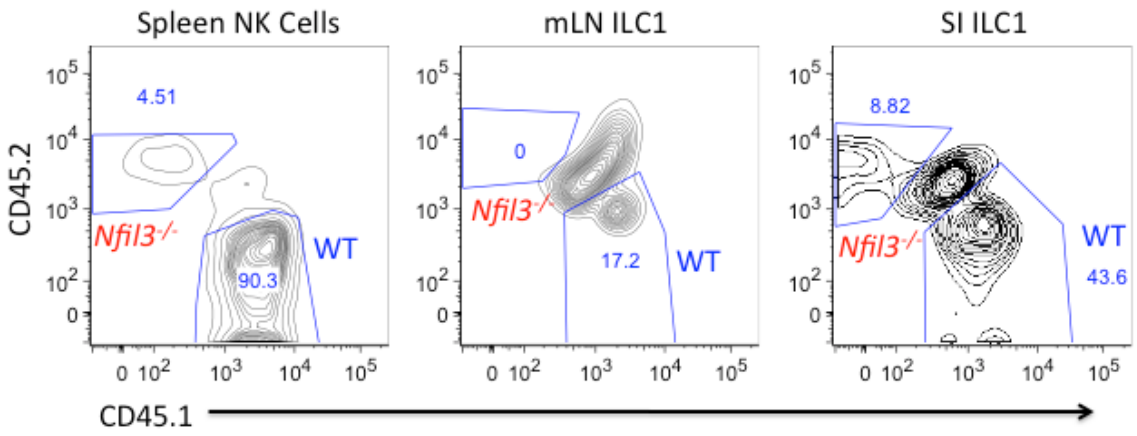


FIGURE 11 | CELL-INTRINSIC REQUIREMENT FOR NFIL3 IN ILC1 AND NK CELL DEVELOPMENT

A 1:1 mixture of *Nfil3*$^{-/-}$ (CD45.2^{+}) and WT (CD45.1^{+}) bone marrow was injected into lethally irradiated recipients. After reconstitution, NK cell (NK1.1^{+} TCR-β^{-}), and ILC1 (lineage^{-} T-bet^{+} IL-7Rα^{+}) chimerism was assessed in indicated organs. CD45.1^{+} x CD45.2^{+} cells represent host population. Data are representative of 3 mice per group.

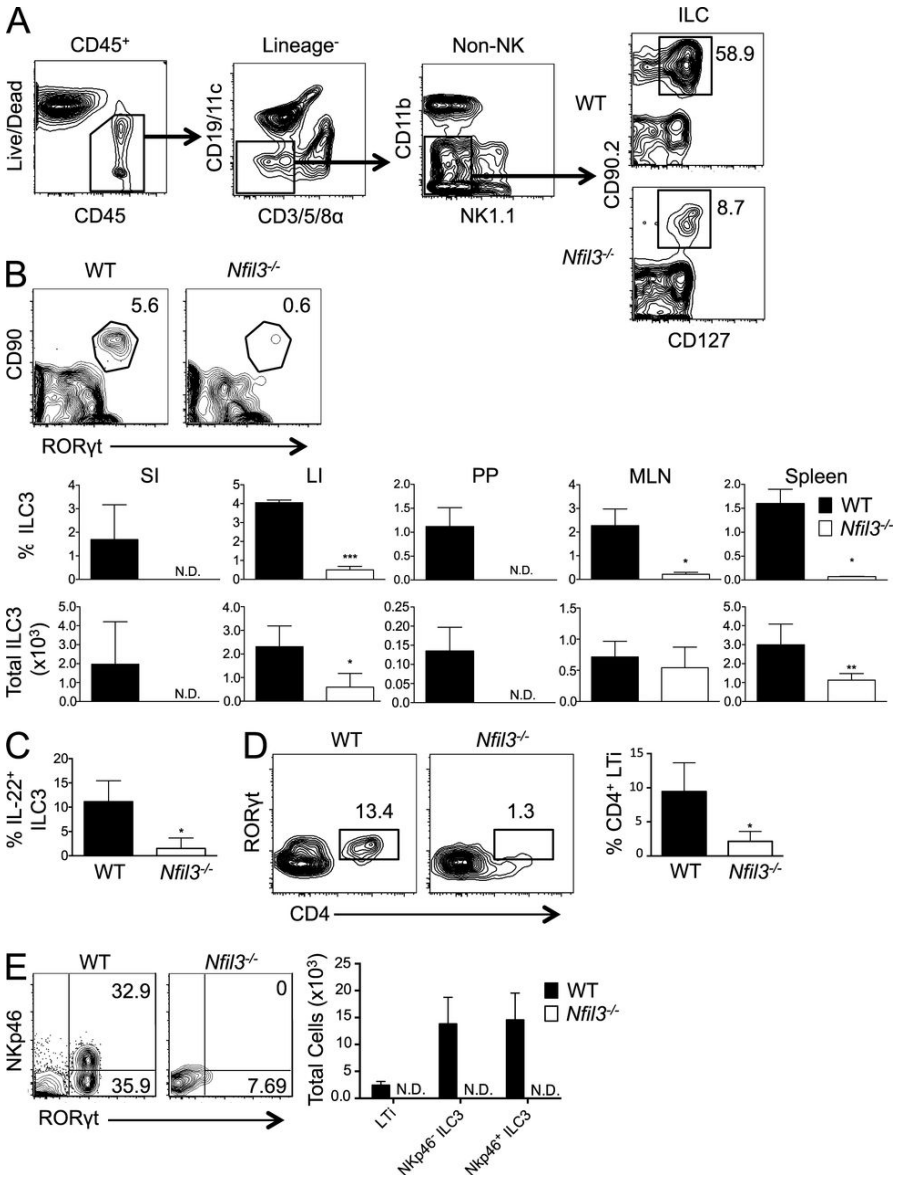


FIGURE 12 | NFIL3 IS REQUIRED FOR INTESTINAL ILC3 AND LTI CELL DEVELOPMENT

(A) Gating strategy for analysis of the total ILC population ($CD90.2^+$ $CD127^+$ cells within the $CD45^+$ $Lineage^-$ population) is shown. (B) Flow cytometric plots show the percentage of $ROR\gamma t^+$ ILC3s within the $CD45^+$ $Lineage^-$ population in the PPs. Graphs show percentage and absolute number of ILC3s within the $CD45^+$ $Lineage^-$ population for SI, LI, PP, MLNs, and spleen from WT and *Nfil3*$^{-/-}$ mice. (C) Graph shows the percentage of IL-22–producing cells within the MLN ILC3 population of WT and *Nfil3*$^{-/-}$ mice after IL-23 stimulation. (D) Percentages of intestinal $CD4^+$ $ROR\gamma t^+$ LTi cells within the total ILC population of WT and *Nfil3*$^{-/-}$ mice are shown. (E) Plots show the percentage of SI $NKp46^-$ and $NKp46^+$ ILC3s, and graph shows absolute numbers of LTi, $NKp46^-$ ILC3, and $NKp46^+$ ILC3. All data are representative of n = 3–5 mice per group, with error bars showing standard deviation, repeated in 2 (panel E) or 4 (panels B-D) independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

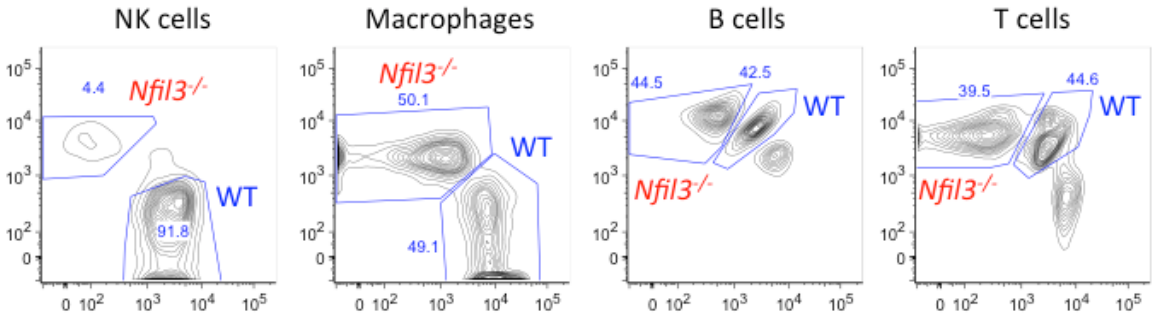


FIGURE 13 | NFIL3 AND WT CHIMERISM IS NORMAL IN T, B, AND MYELOID CELLS

A 1:1 mixture of *Nfil3*$^{-/-}$ (CD45.2^{+}) and WT bone marrow was injected into lethally irradiated recipients. After reconstitution, NK cell (NK1.1^{+} TCR-β^{-}), macrophage (CD11b^{+} F4/80^{+}), B cell (CD19^{+} TCR-β^{-}), and T cell (TCR-β^{+} CD19^{-}) chimerism was determined in the spleen. Ungated populations represent host. Data are representative of two independent experiments, with 3-5 mice per group.

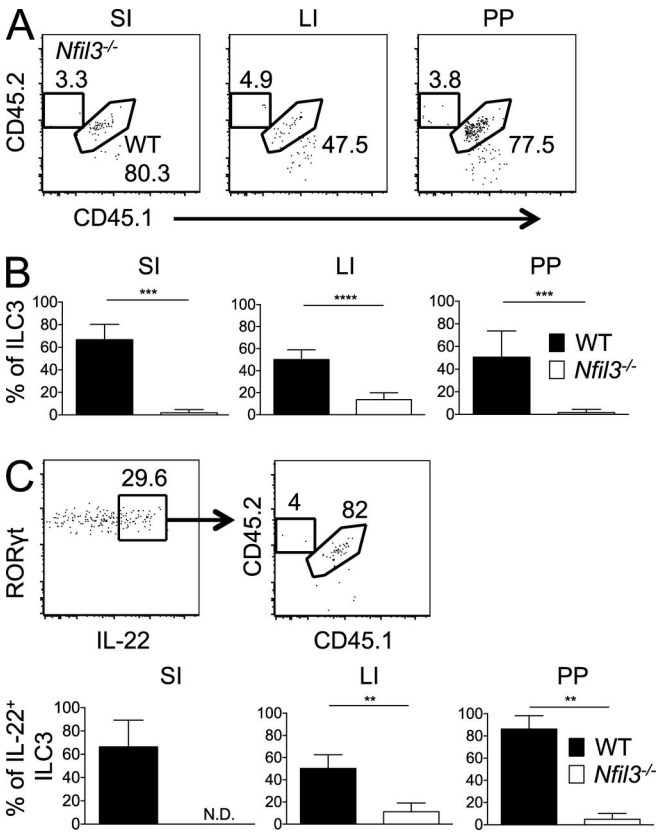


FIGURE 14 | CELL-INTRINSIC ROLE FOR NFIL3 IN DEVELOPMENT OF ILC3S

(A) Percentages of intestinal WT (CD45.1 × 2) and *Nfil3*$^{-/-}$ (CD45.2) ILC3 populations in mixed BM chimeric mice are shown. The CD45.1^{+} population in each plot represents WT host ILC3s. (B) Graphs show percentages of WT and *Nfil3*$^{-/-}$ ILC3 derived from donor BM in the SI, LI, and PP of chimeras. (C) Representative plots and graphs show the percentage of IL-22–producing cells within the intestinal WT and *Nfil3*$^{-/-}$ ILC3 populations after IL-23 stimulation. All data are representative of n = 3–5 mice per group, with error bars showing standard deviation, repeated in 2 (C) or 3 (A and B) independent experiments. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

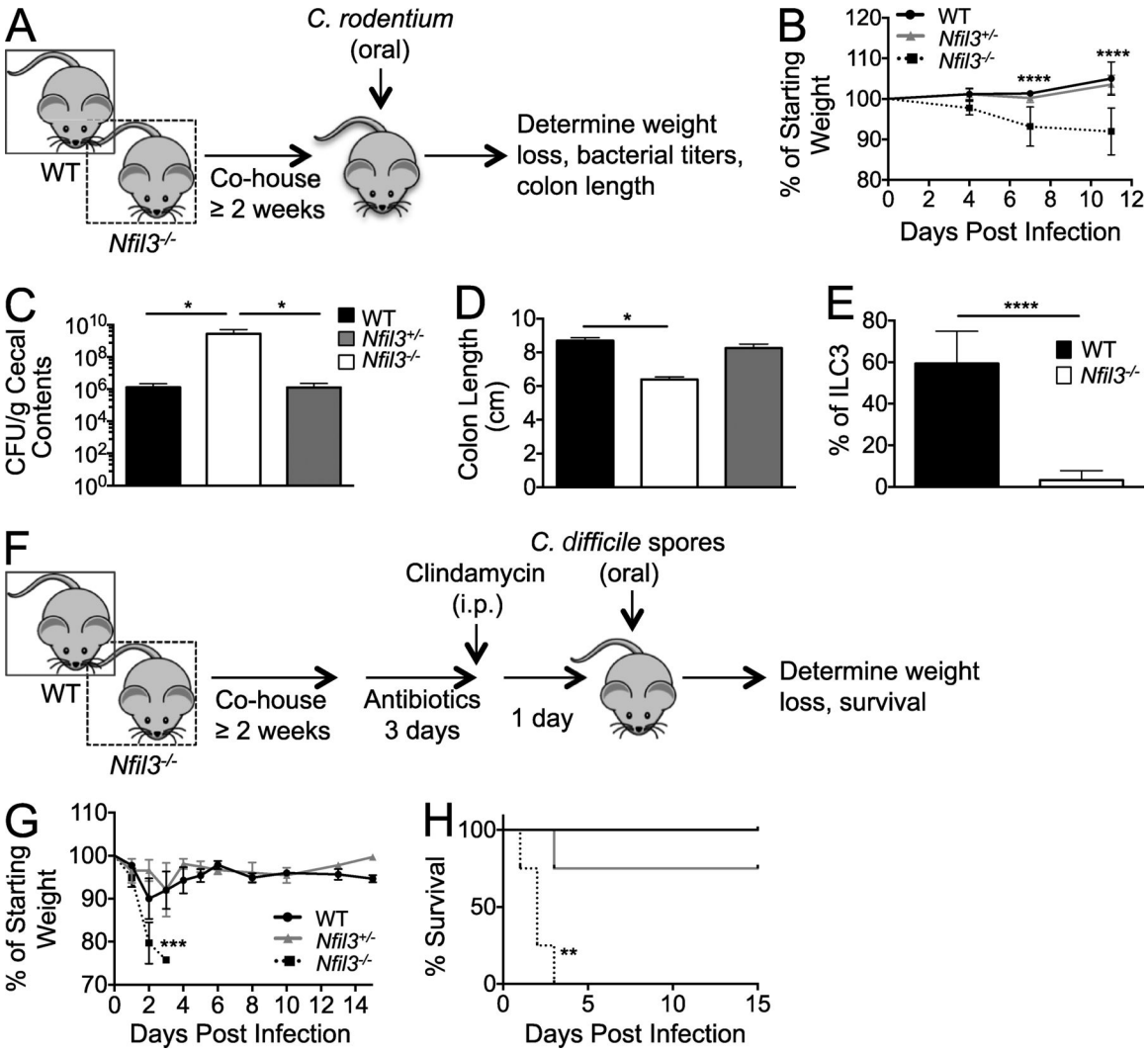


FIGURE 15 | *NFIL3*$^{-/-}$ MICE ARE SUSCEPTIBLE TO INTESTINAL PATHOGENS

(A) Schematic of *C. rodentium* experiment. (B) Body weight of mice from WT, *Nfil3*$^{+/-}$, and *Nfil3*$^{-/-}$ groups was assessed during the course of *C. rodentium* infection. (C and D) Infected WT, *Nfil3*$^{+/-}$, and *Nfil3*$^{-/-}$ mice were sacrificed on day 11 PI, and *C. rodentium* colony forming units (CFU) in cecal content was determined (C), and colon length measured (D). (E) Mixed WT:*Nfil3*$^{-/-}$ chimeric mice were infected with *C. rodentium*, and percentages of WT and *Nfil3*$^{-/-}$ cells within the total colonic ILC3 population on day 2 PI are shown. (F) Schematic of *C. difficile* experiment. (G and H) Body weight (G) and survival (H) of mice from WT, *Nfil3*$^{+/-}$, and *Nfil3*$^{-/-}$ groups was assessed during the course of *C. difficile* infection. All data are representative of n = 3–5 mice per group, with error bars showing standard deviation (B–E) and SEM (G), repeated in 2 independent experiments. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

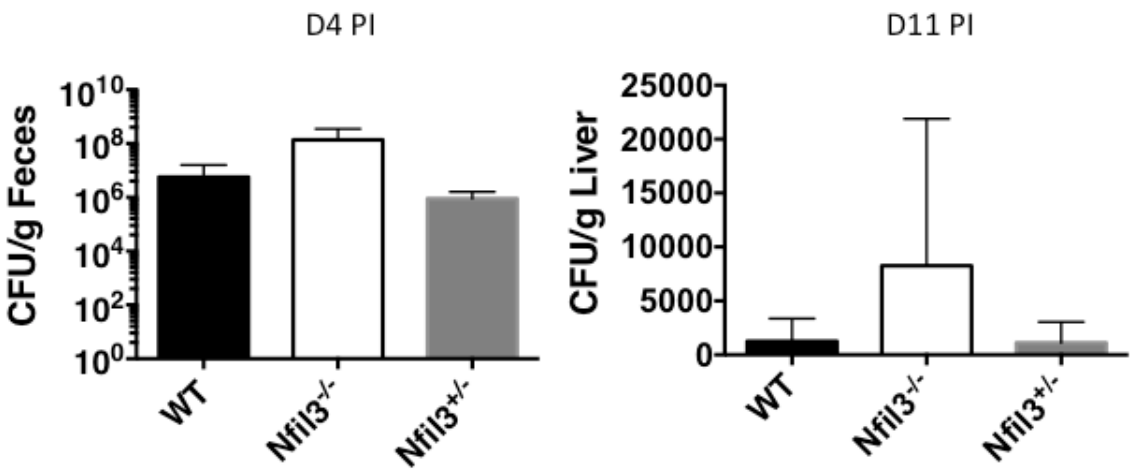


FIGURE 16 | *NFIL3*$^{-/-}$ MICE HAVE HIGHER BACTERIAL TITERS AFTER *C. RODENTIUM* INFECTION

Left: *C. rodentium* CFU per gram of fecal pellets collected from infected mice on day 4 PI. Right: *C. rodentium* CFU per gram of the liver of infected mice on day 11 PI.

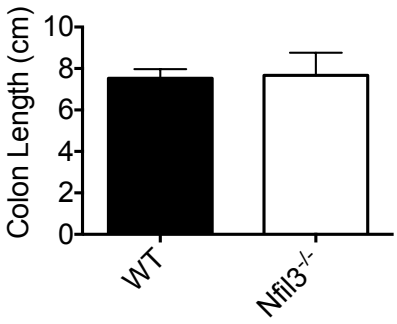


FIGURE 17 | NAÏVE *NFIL3*$^{-/-}$ MICE HAVE NORMAL COLON LENGTH

Colon length of 3 month old female WT (black) and *Nfil3*$^{-/-}$ (white) mice. N = 5 mice/group. Data are representative of two independent experiments.

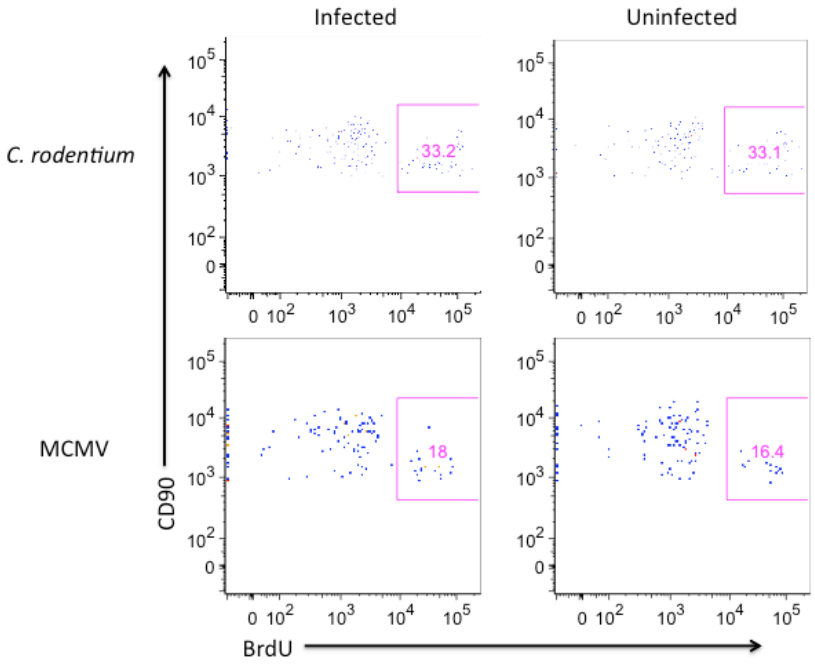


FIGURE 18 | ILC3 DO NOT PROLIFERATE IN RESPONSE TO *C. RODENTIUM* OR MCMV

BrdU incorporation of ILC3 (gated on live, $CD45^+$, lineage negative, ROR-γt$^+$, $CD90^+$) in the Peyer's patches of WT mice infected with *C. rodentium* (day 3 PI, top) or MCMV (day 7 PI, bottom). N = 3 mice/group.

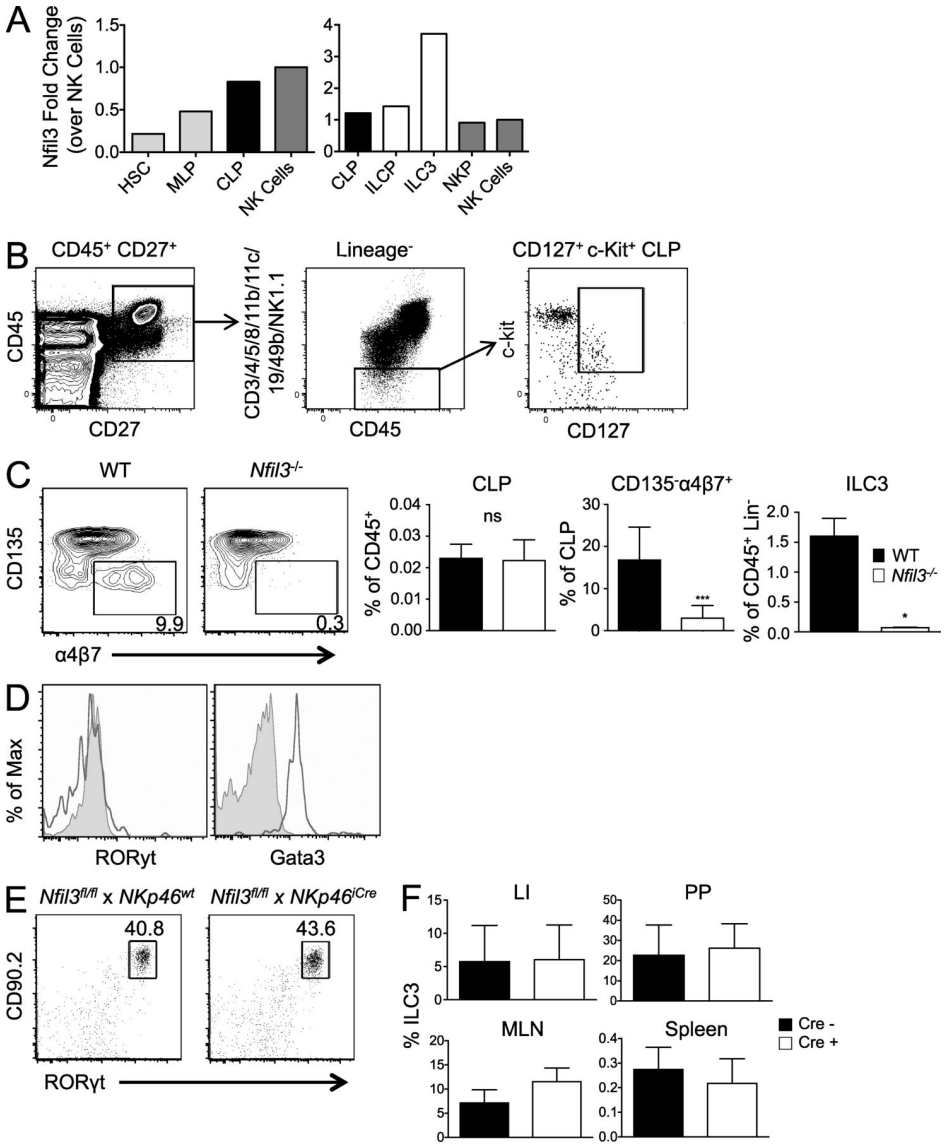


FIGURE 19 | *NFIL3* IS CRITICAL DURING CLP TO ILCP TRANSITION BUT NOT FOR MAINTENANCE OF MATURE ILC3S

(A) Relative *Nfil3* expression was determined by Immgen microarray dataset (left graph) and qRT-PCR (right graph) on indicated cell populations. Data are shown as fold change relative to *Nfil3* expression in NK cells. (B) Gating strategy shown for analysis of CLP ($CD127^+$ $c\text{-}Kit^+$ cells within the $CD45^+$ $CD27^+$ $Lineage^-$ population) in BM. (C) Plots show percentage of BM ILCP ($CD135^-$ $\alpha4\beta7^+$) within the CLP population. Graphs show percentages of CLP and ILCP in BM and ILC3 in the spleen of WT and $Nfil3^{-/-}$ mice. (D) Histograms show expression of RORγt and Gata3 in CLP (tinted) and ILCP (black line). (E) Percentages of intestinal ILC3s in $Nfil3^{fl/fl}$ × $Nkp46^{iCre}$ mice and littermate control (without Cre) are shown. (F) Graphs show percentage of ILC3s in the indicated tissues from $Nfil3^{fl/fl}$ × $Nkp46^{iCre}$ mice and littermate controls. Data are representative of n = 3–5 mice per group, with error bars showing standard deviation, repeated in 2 (C–F) or 3 (A) independent experiments. *, P ≤ 0.05; ***, P ≤ 0.001.

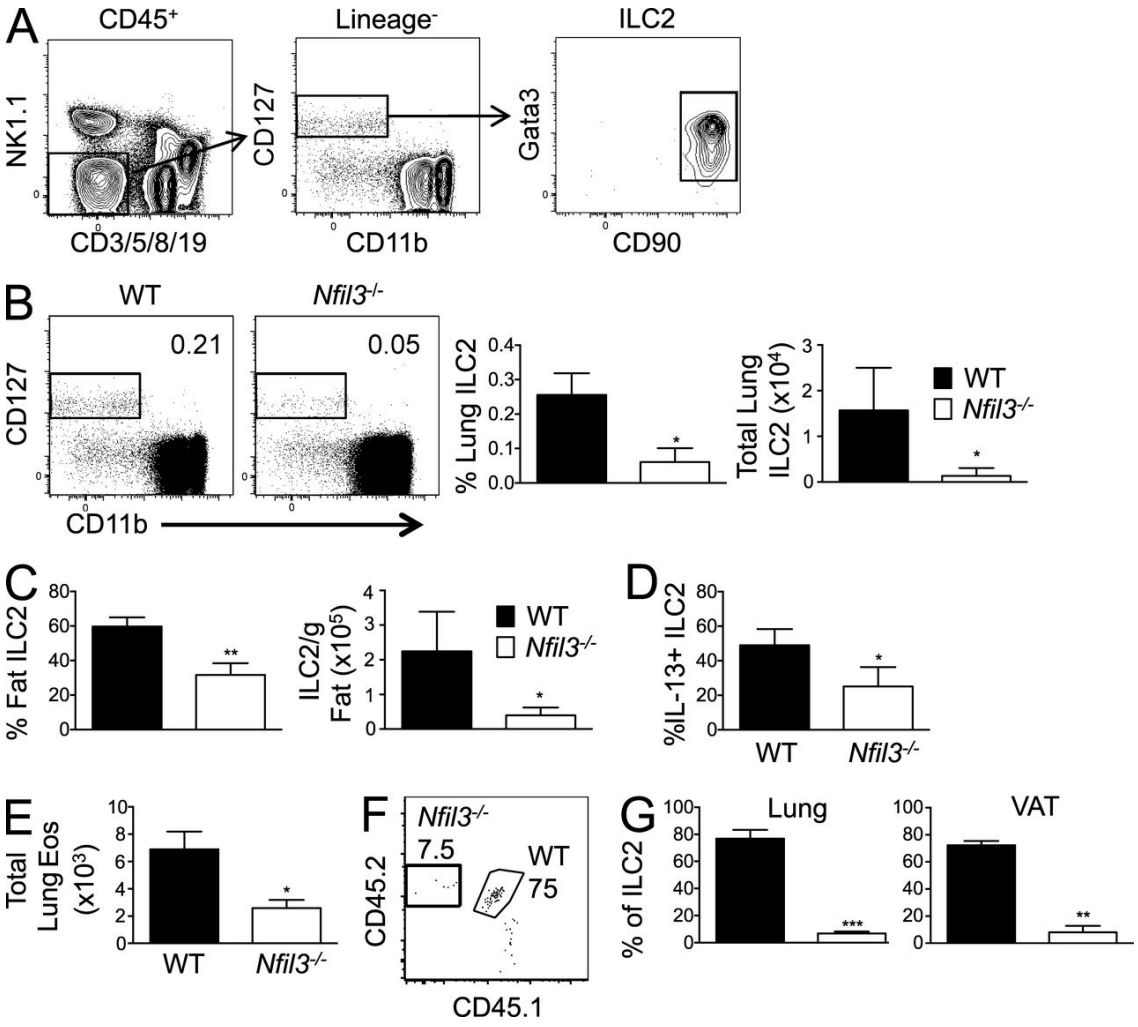


FIGURE 20 | *NFIL3*$^{-/-}$ MICE ARE DEFICIENT IN LUNG AND FAT ILC2S AND EOSINOPHILS

(A) Gating strategy shown for analysis of lung ILC2 (CD90.2$^+$ Gata3$^+$ cells within the CD45$^+$ Lineage$^-$ population). (B) Percentage (of CD45$^+$) and absolute number of ILC2s in lungs of WT and *Nfil3*$^{-/-}$ mice are shown in plots and graph. (C and D) Graphs show total ILC2s (C) and IL-13–secreting ILC2s (after stimulation with PMA + Ionomycin; D) in VAT from WT and *Nfil3*$^{-/-}$ mice. (E) Absolute number of eosinophils (Lin$^-$ CD45$^+$ CD90$^-$ NK1.1$^-$ CD11b$^+$ SiglecF$^+$) in lungs of WT and *Nfil3*$^{-/-}$ mice is shown. (F) Percentages of WT (CD45.1 × 2) and *Nfil3*$^{-/-}$ (CD45.2) lung ILC2 populations from mixed BM chimeric mice are shown. The CD45.1$^+$ population in each plot represents WT host ILC2s. (G) Graph shows percentages of WT and *Nfil3*$^{-/-}$ lung and VAT ILC2s derived from donor BM in chimeric mice. All data are representative of n = 3–5 mice per group, with error bars showing standard deviation, repeated in 2 (E), 3 (F and G), or 4 (B–D) independent experiments. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

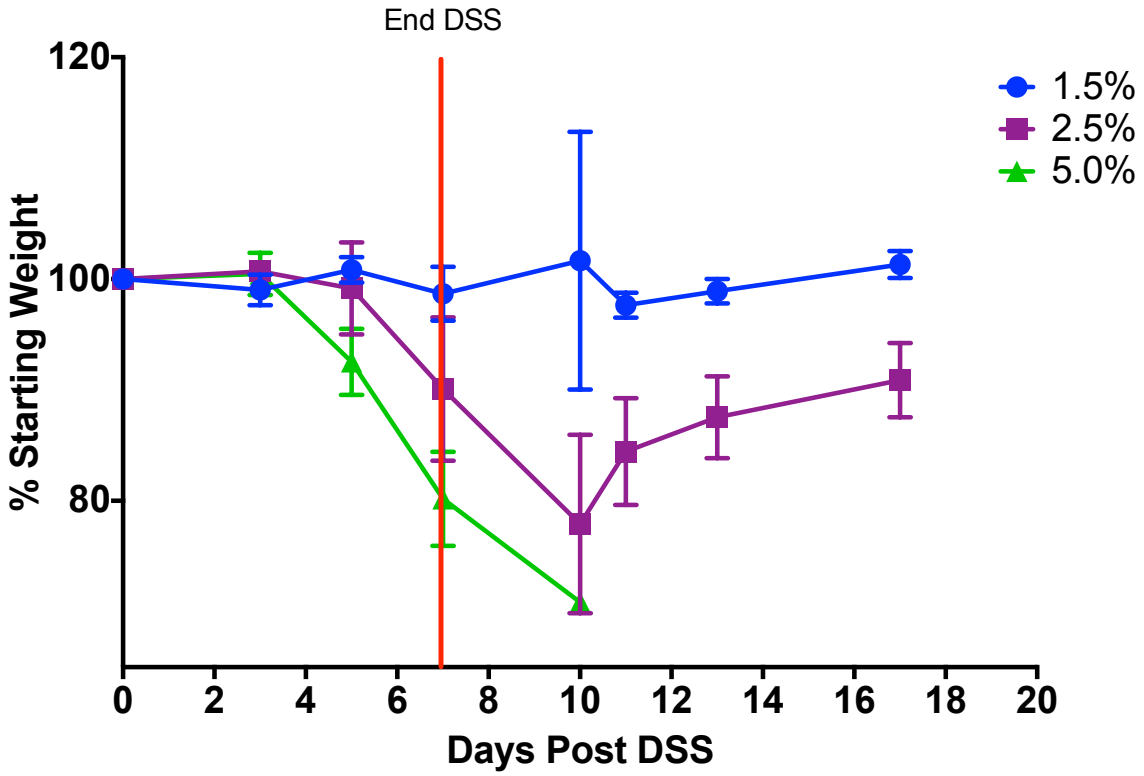


FIGURE 21 | TITRATION OF DSS DOSE IN WT MICE

WT mice were given 1.5% (blue), 2.5% (purple), or 5.0% (green) DSS in drinking water for 7 days, followed by standard water. Mice were monitored for weight loss during DSS treatment and subsequent recovery. N = 4-5 mice per group.

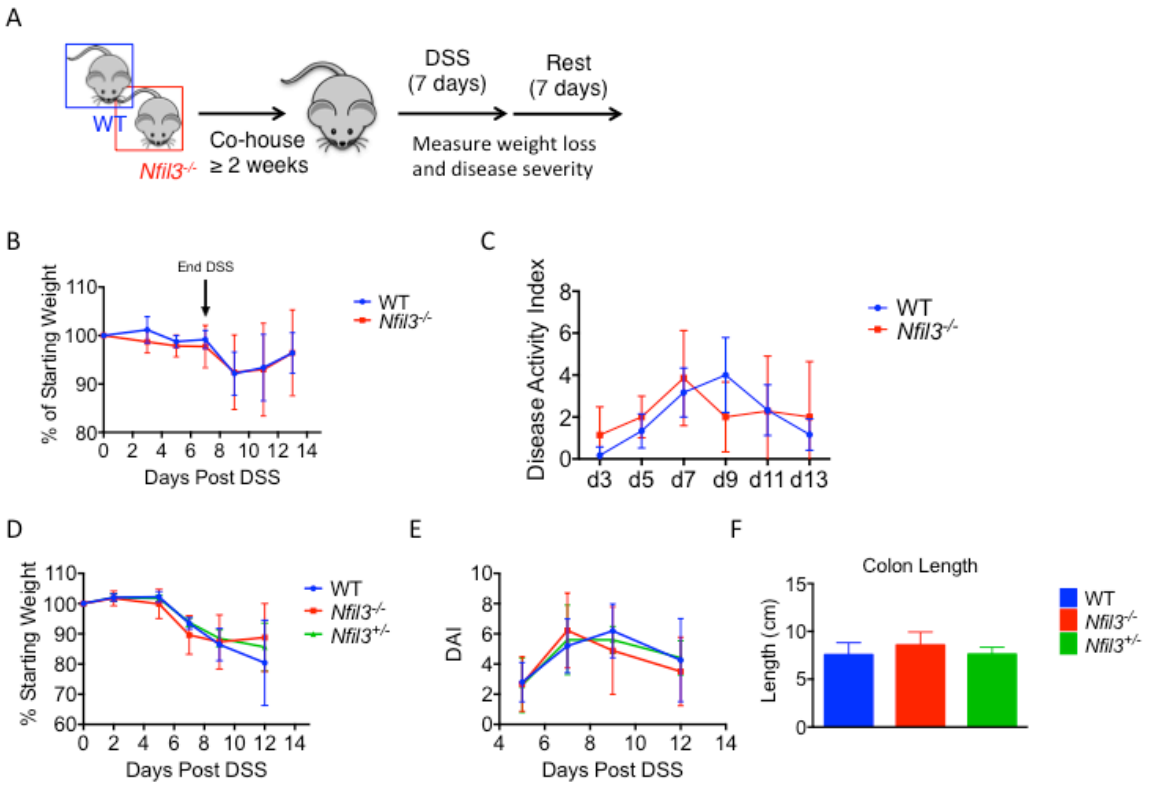


FIGURE 22 | *NFIL3*$^{-/-}$ MICE ARE NOT MORE SUSCEPTIBLE TO LOW DOSES OF DSS

(A) *Nfil3*$^{-/-}$ mice and their WT littermates were cohoused for at least two weeks before being given 1.5% (B,C) or 1.8% (D-F) DSS in drinking water for 7 days followed by 5-7 days of standard water. Mice were monitored for weight loss (B, D) and disease activity index (C, E) throughout DSS treatment and rest. Disease activity index is a combined measure of disease severity using a blood, stool, and weight score. Blood scoring: 0 = no blood, 1 = hemoccult +, 2 = visible blood in stool, 3 = blood on anus. Stool scoring: 0 = normal stool, 2 = loose stool, 3 = diarrhea. Weight scoring: weight loss: 0 (no loss), 1 (1-5%), 2 (5-10%), 3 (10-20%), and 4 (>20%). Colon length of mice given 1.8% DSS was also measured at the endpoint (F). N= 5-9 mice/group. Data are displayed as mean with error bars showing standard deviation.

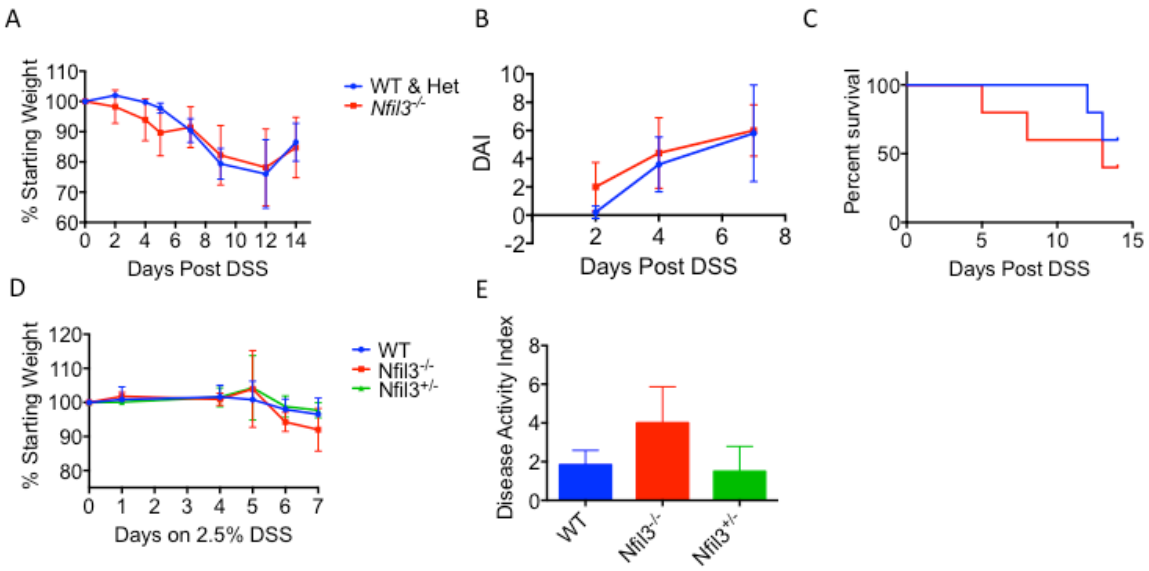


FIGURE 23 | *NFIL3*$^{-/-}$ MICE ARE SLIGHTLY MORE SUSCEPTIBLE TO MODERATE DOSES OF DSS

Nfil3$^{-/-}$ mice and their WT and heterozygous littermates were cohoused for at least two weeks before being given 2.0% (A-C) or 2.5% (D-E) DSS in drinking water for 7 days followed by 7 days of standard water (A-C) or sacrifice (D-E). Mice were monitored for weight loss (A,D) and disease activity index throughout DSS treatment (B) or after 7 days on DSS (E). Disease activity index is a combined measure of disease severity using a blood, stool, and weight score. Blood scoring: 0 = no blood, 1 = hemoccult +, 2 = visible blood in stool, 3 = blood on anus. Stool scoring: 0 = normal stool, 2 = loose stool, 3 = diarrhea. Weight scoring: weight loss: 0 (no loss), 1 (1-5%), 2 (5-10%), 3 (10-20%), and 4 (>20%). Mice given 2.0% DSS were also monitored for morbidity (C). N= 3-6 mice/group. Data are displayed as mean with error bars showing standard deviation.

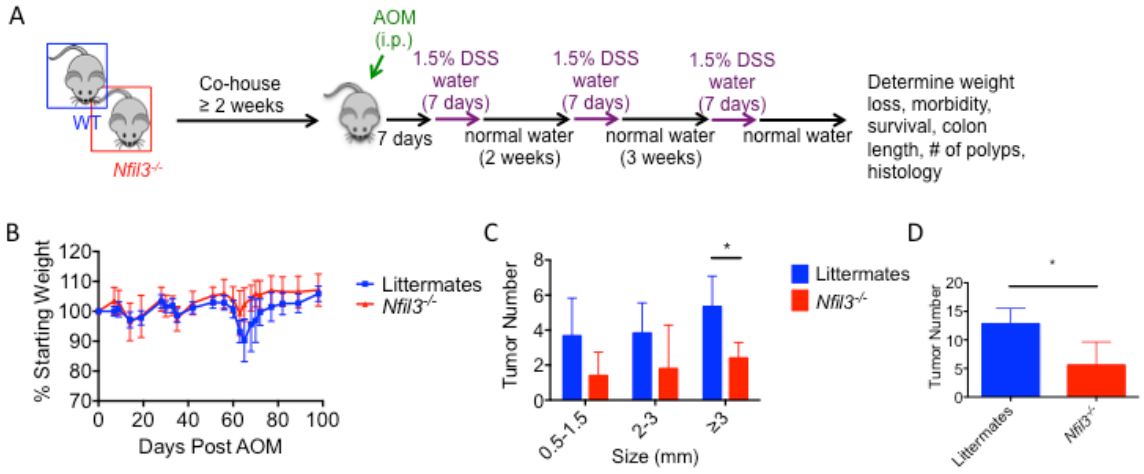


FIGURE 24 | *NFIL3*$^{-/-}$ MICE ARE LESS SUSCEPTIBLE TO AOM/DSS COLON POLYPS

(A) *Nfil3*$^{-/-}$ mice were co-housed with WT and heterozygous littermates for at least two weeks before being injected with 10 mg/kg AOM intraperitonealy (i.p.). Mice were then allowed to rest for 7 days, followed by successive cycles of 1.5% DSS in the drinking water and rest periods. Weight loss was measured over the course of DSS treatment (B) and the mice were euthanized and number of colon polyps were counted and measured using methelyne blue staining 98 days after AOM treatment. *Nfil3*$^{-/-}$ mice had significantly fewer large tumors (≥3 mm) than littermates (C) and significantly fewer tumors overall (D). All data are representative of N = 4–7 mice per group, with error bars showing standard deviation, repeated in 2 independent experiments. *, P ≤ 0.05.

III. Discussion

In summary, we have demonstrated that Nfil3 is a critical transcription factor promoting all ILC lineages. Mice deficient in Nfil3 lack group 1, group 2, and group 3 ILCs, likely due to the loss of the αLP early ILC progenitor. Our findings suggest Nfil3 may act as a "master regulator" for the ILC lineage. However, exactly how Nfil3 fits into the larger network of transcription factors governing ILC development requires further study. For example, while *Nfil3*$^{-/-}$ mice display a severe ILC deficiency in all organs we studied, some residual ILCs do appear to develop, and the number and percentage varies between organs. Whether these represent a unique developmental lineage of ILC or ILCs that have managed to bypass the requirement for Nfil3 in some other way is currently unknown. One hypothesis for the presence of these Nfil3-independent ILCs is that baseline inflammation in different tissue types may be able to drive out a small percentage of ILCs. It has been shown that MCMV infection and the resulting activating and pro-inflammatory environment is able to drive out Nfil3-independent NK cells (Firth et al. 2013). Furthermore, the relationship between Nfil3 and other early ILC factors remains unclear. The transcription factors Tox and Id2 are thought to be downstream of Nfil3. However, as we and others have shown, *Nfil3*$^{-/-}$ mice develop lymph nodes in the absence of ILC precursors (Seillet, Rankin, et al. 2014; Xu et al. 2015), while Tox and Id2 deficient mice lack both ILC precursors and lymph nodes (Yokota et al. 1999; Aliahmad et al. 2010). This suggests a putatitive second factor that may act in concert with or in place of Nfil3 to regulate Tox and Id2 (Seehus and Kaye 2015). Alternatively, Nfil3, Tox,

and Id2 may be regulated differently during fetal development than they are in an adult. In support of this idea, more CHILPs were found in fetal livers of *Nfil3*$^{-/-}$ mice than in adult bone marrow (Xu et al. 2015). Furthermore, a novel ILC precursor has been identified in the fetal intestine that may seed ILC populations during early development (Bando et al. 2015), but it has not yet been reported whether this population depends on Nfil3.

Our findings further suggest that Nfil3 expression is critical for maintaining early immunity at mucosal surfaces. In the *C. rodentium* model, it has been well established that ILC3s are critical for protection (Cella et al. 2009; Qiu et al. 2012; Satoh-Takayama et al. 2008; Sonnenberg et al. 2012; Sonnenberg et al. 2011), consistent with our findings that *Nfil3*$^{-/-}$ mice, lacking all ILC subsets, are more susceptible to the disease. NK cells have also been reported to help control bacterial loads and dissemination in *C. rodentium* infected mice (Hall et al. 2013), which may also be contributing to our phenotype. In the *C. difficile* model where we see severe weight loss and morbidity in *Nfil3*$^{-/-}$ mice, recent work has shown that it is mainly ILC1s and their production of IFN-γ, with some contribution from ILC3s, that mediate protection (Abt et al. 2015).

The loss of ILCs in *Nfil3*$^{-/-}$ mice may also render them susceptible to spontaneous colitis (Kobayashi et al. 2014). Consistent with this finding is a report that colonic mononuclear cells from Crohn's disease and ulcerative colitis patients express lower levels of Nfil3 compared to noninflammatory controls (Kobayashi et al. 2011). Genome-wide association studies have also identified the Nfil3 locus as a risk factor in human inflammatory bowel disease (IBD)

(Jostins et al. 2012). However, we surprisingly see only very modest increased susceptibility to DSS-induced colitis in *Nfil3*$^{-/-}$ mice. One potential reason for this is that *Nfil3*$^{-/-}$ mice of different ages may have differences in their susceptibility to colitis. Though Kobayashi et al. showed that *Nfil3*$^{-/-}$ mice have shorter colons that WT mice at 12-18 weeks of age, the majority of their mice did not display rectal prolapse until around 35 weeks of age (Kobayashi et al. 2014). The mice used in our DSS studies were between 12 and 20 weeks of age and we do not observe differences in colon length at that age. It is therefore possible that susceptibility to colitis is an age-related phenotype in *Nfil3*$^{-/-}$ mice. It is also likely that microbiota differences between facilities contributes to colitis susceptibility, given that the spontaneous colitis in the same strain of *Nfil3*$^{-/-}$ mice has varying penetrance and severeity between different institutions (Kobayashi et al. 2014). Furthermore, it is possible that inflammation induced by DSS treatment is able to drive out some Nfil3-independent ILCs, masking any phenotype due to ILC-deficiency. On the other hand, it has been suggested that chronic DSS-induced colitis is partially mediated by a shift in the balance between protective IL-22-producing ILC3 and pathogenic IFN-γ-producing ILC1 (Kimura et al. 2012). Therefore, it is possible that *Nfil3*$^{-/-}$ mice do not show increased pathology compared to WT littermates because they lack both the protective and the pathogenic ILCs. Conversely, *Nfil3*$^{-/-}$ mice appear less susceptible to colorectal cancer in a DSS-induced colitis-driven model. This is potentially due to their lack of ILC3s, consistent with a study showing that ILC-derived IL-22 is pro-tumorigenic in a related model of colon cancer (Kirchberger et al. 2013). IL-22 has also been shown to be tumor

promoting in human colon cancer patients (Jiang et al. 2013), likely through STAT3, a known tumor promoter (Yu et al. 2009).

The status of Nfil3 as an early developmental factor for all ILCs makes it a crucial avenue for future study. Further investigation into both genes regulating Nfil3 and targets of Nfil3 will help identify and elucidate the relationships between the ILC lineages. The importance of Nfil3 in so many disease models further indicates its potential as a risk factor or therapeutic target in human disease.

IV. Materials and Methods

1. Mice[2]

Wildtype C57BL/6 (B6), congenic (CD45.1 and CD45.1xCD45.2), *Nfil3*$^{-/-}$ (Kashiwada et al. 2010), *Nfil3*$^{fl/fl}$ (Motomura et al. 2011), and *Nkp46*^{iCre} (Narni-Mancinelli et al. 2011) mice were bred and maintained at MSKCC. Mice were housed and maintained according to Memorial Sloan Kettering Cancer Center guidelines, and all experiments were carried out in accordance with MSKCC Institutional Animal Care and Use Committee approval and institutional guidelines. Mixed bone marrow chimeric mice were generated, as previously described (Sun et al. 2009). In oral infection studies, mice were co-housed for a period of 2-3 weeks prior to bacteria challenge in order to normalize bacteria flora between experimental groups. Wildtype controls were age and sex-matched C57BL/6 mice or WT littermates, as indicated.

2. Bacterial Infections and Titers[2]

In *C. rodentium studies*, mice were inoculated by oral gavage with 108 CFU (in 200 µL) of an overnight LB culture of *C. rodentium* (strain DBS100). Infected mice were assessed for body weight, signs of morbidity, and bacterial titers. To determine *C. rodentium* titers, fecal or cecal contents were mechanically homogenized in PBS and 10-fold serial dilutions cultured overnight on MacConkey's agar, as previously described (Sonnenberg et al. 2011). In *C. difficile* studies, mice were treated with antibiotic water (0.25g/L metronidazole, 0.25g/L neomycin, and 0.25g/L vancomycin) from day -6 to -3 and received 200 µg clindamycin i.p. on day −1 prior to infection with 200 CFU of *C. difficile* spores (strain VPI 10463) by oral gavage. Infected mice were assessed for body weight and signs of morbidity (Buffie et al. 2012).

3. Viral Infection

MCMV (Smith strain) was obtained from L. Lanier (University of California San Francisco). MCMV was twice passaged through BALB/c hosts and stocks were generated by douncing salivary glands of mice 3 weeks after MCMV infection.

Mice were infected by i.p. injection of approximately 7.5×10^3 PFU of MCMV in 500 uL of PBS.

4. BrdU Injection and Staining

Mice were given BrdU (5-Bromo-2´-Deoxyuridine) (Sigma) on day 2 (*C. rodentium*, 1.0 ug) or day 3 (MCMV, 1.5 ug) post-infection by i.p. injection. Mice were euthanized and ILCs from day 3 (*C. rodentium*) and day 7 (MCMV) infected mice were stained with an anti-BrdU mAb (BD Biosciences).

5. Isolation of ILC Subsets and *Ex Vivo* Stimulation[2]

Spleens, mesenteric lymph nodes, and Peyer's patches were mechanically crushed into single cell suspensions. Lungs, intestines, and fat were digested in collagenase type 4 (Worthington), collagenase D (Roche), and collagenase type 2 (Worthington) respectively. To assess production of cytokines, ILC2 and ILC3 cells were stimulated for 3 hours at 37ºC in complete RPMI + 10% FBS with 1:1000 Brefeldin A (BD), 1:1000 2-mercaptoethanol (Sigma), and 40 ng/mL IL-23 (for ILC3 stimulation) or 0.1ug/mL PMA + 1ug/mL ionomycin (for ILC2 stimulation), followed by intracellular staining. Unstimulated controls (media only) were used to determine gating strategy for flow cytometric plots in figures.

6. Flow Cytometry[2]

Single cell suspensions were generated from indicated organs and incubated with the anti-Fc receptor antibody 2.4G2 prior to staining with indicated monoclonal antibodies (Biolegend, eBioscience, BD) for 20 min on ice. In certain experiments, staining was performed on transcription factors and intracellular cytokines using the FoxP3 staining kit (eBioscience) according to manufacturer protocols. Lineage-negative cells are defined as lacking surface CD3, CD4, CD5, CD8, CD11b, CD11c, CD19, CD49b, Gr-1, and NK1.1. Samples were acquired using an LSRII flow cytometer with FACSDiva software (BD), and analysis was performed with FlowJo 9.6 software (TreeStar).

7. Quantitative Real Time-PCR[2]

Bone marrow CLP (lin^- $c\text{-}kit^+$ $sca1^+$ $flt3^+$ $IL\text{-}7Ra^+$), and ILCP (lin- $c\text{-}kit^+$ $sca1^+$ $flt3^-$ $IL\text{-}7Ra^+$ $\alpha4\beta7^+$), gut ILC3 (lin^- $Rorc^+$ $IL\text{-}7Ra^+$) were sorted to ~99% purity on an

Aria II cytometer (BD Biosciences). Cell lysis was subsequently carried out using Tri-Reagent (Ambion) and RNA was purified using the Qiagen RNeasy kit (with on-column DNase I treatment), and MuLV reverse transcriptase and oligo(dT)16 primers (Applied Biosystems) were used for cDNA synthesis. iQ Sybr Green SuperMix (BioRad) was used for qRT-PCR. Data were normalized to expression of β-actin and expressed as relative target abundance via the ΔΔCt method, where Ct (threshold cycle) is the cycle number at which the amplification curve intersects the threshold value. The primer sets used for qRT-PCR are the following: Nfil3 forward, 5′-AATTCATTCCGGACGAGAAG-3′; Nfil3 reverse, 5′-CGATCAGCTTGTTCTCCAAA-3′; β-actin forward, 5′-TGCGTGACATCAAAGAGAAG-3′; and β-actin reverse, 5′-CGGATGTCAACGTCACACTT-3′.

8. Statistical Analysis[2]

Results are expressed as mean with error bars showing ± SD unless otherwise indicated. Data were analyzed using two-tailed unpaired Students t-test with Welch's correction or one-way ANOVA (with multiple comparisons where applicable). All analyses were performed using Prism 5.0b (GraphPad) software, and differences were considered significant when $p \leq 0.05$. Symbols in figures denote statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

9. AOM/DSS Treatment and Tumor Counting

Mice were injected with 10 mg/kg azoxymethane in PBS (AOM, Sigma Aldrich) by intraperitoneal injection. Indicated percentages of dextran sulfate sodium

(DSS, Affymetrix) by weight were provided in drinking water *ad libitum*. Stool samples were tested for occult blood using Hemoccult test kit (Beckman Coulter). Colons were removed, flushed with cold PBS, linearized, and fixed in 10% formalin for 4 hours at room temperature, then moved to 70% ethanol. For polyp counting and measurement, colon sections were stained in methylene blue and examined under a microscope.

Chapter 3: TGF-β is Dispensable for NK cell Development

I. Introduction

NK cells are controlled by a balance of activating and inhibitory receptors as well as pro and anti-inflammatory cytokines. Activating receptors or pro-inflammatory cytokines trigger NK cells to secrete interferon (IFN)-γ (Stetson et al. 2003) or lytic molecules such as perforin and granzymes (Smyth et al. 2005). Though these activating signals have been well studied, much less is known about how anti-inflammatory signals, such as the regulatory cytokine TGF-β, might influence NK cell development and function.

1. TGF-β Signaling

TGF-β is a cytokine important in controlling many immune cell types. TGF-β1$^{-/-}$ mice develop a severe autoimmune inflammatory disease (Shull et al. 1992; Kulkarni et al. 1993). The TGF-β superfamily consists of three homologous isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3, as well as bone morphogenic proteins (BMP), activins, and other groups of factors (Chang et al. 2002; Govinden and Bhoola 2003). TGF-β is secreted in a latent form, as a homodimer non-covalently associated with a homodimer of a protein called latency associated peptide (LAP). This complex is also sometimes associated with latent-TGF-β-binding protein (LTBP) (Annes et al. 2003), which can target TGF-β to the extracellular matrix (Li and Flavell 2008). Latent TGF-β can be activated *in vivo* by interaction with the protease plasmin, the protein thrombospondin-1, or

αVβ6 integrin, which results in the removal of LAP (Nunes et al. 1995; Crawford et al. 1998; Yehualaeshet et al. 1999; Munger et al. 1999; Annes et al. 2004). Mice with LAP that is unable to bind integrins display the autoimmune phenotype of TGF-β deficient mice, demonstrating the critical role of integrins in TGF-β activation *in vivo* (Yang et al. 2007). More specifically, integrins expressed on dendritic cells (DCs) and other myeloid cells are thought to play a critical role in activating TGF-β and preventing autoimmunity and colitis (Travis et al. 2007; Lacy-Hulbert et al. 2007). Expression of αVβ8 integrin on regulatory T cells is also thought to be one mechanism by which they suppress T cell inflammatory responses (Worthington et al. 2015). Active TGF-β mediates canonical signaling by binding to a type II TGF-β receptor (TGF-βRII), leading to formation of a tetrameric receptor complex consisting of two type II receptors and two type I (TGF-βRI) receptors (Figure 25) (Wrana et al. 1994). This complexation allows the constitutively active kinase domain of TGF-βRII to phosphorylate TGF-βRI, stabilizing its active conformation and allowing binding and phosphorylization of a receptor regulated Smad (R-Smad e.g. Smad2, Smad3) (Huse et al. 2001). Activated R-Smad then accumulates in the nucleus and binds common mediatior Smad (co-Smad) Smad4, allowing it to interact with a DNA-binding partner and modulate transcription (Massagué et al. 2005). Non-canonical Smad-independent TGF-β signaling pathways also exist, where TGF-β activates the mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and other pathways (Li and Flavell 2008). TGF-βRII has also been reported to directly phosphorylate targets such as PAR6 (Massague 2012).

2. TGF-β Regulation of T cells

TGF-β inhibits the differentiation of naïve $CD4^+$ T cells into T_h1 or T_h2 cells, and prevents $CD8^+$ T cells from acquiring cytotoxic capabilities (Gorelik and Flavell 2002; Ranges et al. 1987; Sad and Mosmann 1994; Swain et al. 1991; Li et al. 2007). Mice expressing a dominant-negative TGF-βRII develop autoimmunity due to spontaneous T cell activation (Gorelik and Flavell 2000), and mice with a T-cell specific TGF-βRII deletion develop severe autoimmune disease due to differentiation of T cells into cytotoxic $CD8^+$ and T_h1 type cells (Marie et al. 2006). Conversely, TGF-β has been shown to support differentiation of $CD8^+$ T cells by promoting expression of IL-7Rα (Ouyang et al. 2013; Li et al. 2006). TGF-β inhibits T_h2 development by inhibiting expression of the key T_h2 transcripitional activator Gata3 (Gorelik et al. 2000; Heath et al. 2000). The mechanism for inhibition of T_h1 development is less clear, as it does not occur in all contexts (Gorelik and Flavell 2002). For example, early production of IFN-γ can release the block on T_h1 development (Sad and Mosmann 1994). TGF-β has been shown to suppress expression of the IL-12 receptor β2-chain (IL-12Rβ2), thereby potentially inhibiting a critical signaling pathway for T_h1 development (Gorham et al. 1998). TGF-β also suppresses STAT3 and STAT4 activation downstream of IL-12 (Bright and Sriram 1998). However, more recent work has shown that TGF-β mediated suppression of T_h1 differentiation is actually due to suppression of the critical T_h1 transcription factor T-bet (Gorelik et al. 2002). The cellular source of TGF-β can also be crucial to determine its effects. It has been shown that autocrine TGF-β is specifically required for differentiation of T_h17 cells

(Li et al. 2007; Gutcher et al. 2011).

It has been suggested that these “non-effector” T cells that develop in the presence of TGF-β are central memory T cells (Gorelik and Flavell 2002). TGF-β has been shown to selectively promote apoptosis of short-lived effector cells (SLECs) but not memory precursor effector cells (MPECs) in mice infected with *Listeria monocytogenes*, potentially by antagonizing the IL-15 dependent upregulation of the anti-apoptotic molecule Bcl-2 (Sanjabi et al. 2009). Bcl-2 expression a the peak of *L. monocytogenes* or LCMV infection correlates with memory cell potential in $CD8^+$ T cells (Dunkle et al. 2013; Grayson et al. 2000). Furthermore, TGF-β has been shown to be required throughout the lifetime of memory T cells, as deletion of the receptor both early and late during infection significantly shifted the population towards SLECs over MPECs (Ma and Zhang 2015). This is thought to be due to alterations in their transcriptional program, with T cells deleted of TGF-β receptor during infection showing higher expression of the memory T cell associated transcription factor Eomes and lower expression of the effector associated transcription factor T-bet compared to controls (Ma and Zhang 2015). It has also been shown that TGF-β signaling is increased in virus-specific $CD8^+$ T cells in chronic infections compared to acute infections and that TGF-β induces apoptosis in effector cells (Tinoco et al. 2009). Similarly, TGF-βRII-deficient $CD8^+$ T cells have a defect in generating gut-resident memory cells, likely due to decreased expression of intestinal homing and retention markers such as αEβ7 integrins and CD69 (Zhang and Bevan 2013) Conversely, another study demonstrated that Smad4 signaling independent of the TGF-βRII

was required for central memory $CD8^+$ T cell development (Hu et al. 2015). Futhermore, another group showed that blocking TGF-β signaling during T cell stimulation results in a higher percentage of central memory phenotype T cells (Takai et al. 2013). The precise role of TGF-β in the generation of memory T cells is therefore still somewhat unclear.

3. TGF-β Regulation of NK Cells

Though there are many studies investigating the role of TGF-β in regulating T cell development and function (Li and Flavell 2008), there have been comparatively few studying TGF-β in NK cells. Several cell types are thought to suppress NK cell function by secreting TGF-β, including regulatory T cells, platelets, tumor cells, and myeloid derived suppressor cells (Ghiringhelli et al. 2005; Kopp et al. 2009; Dasgupta et al. 2005; Szczepanski et al. 2011; Wahl et al. 2006; Li et al. 2009). NK cells themselves can also secrete TGF-β (Wahl et al. 2006; Gray et al. 1994). *In vitro*, it has been shown that TGF-β can suppress NK cell expression of activating receptors (Wilson et al. 2011; Kopp et al. 2009; Castriconi et al. 2003; Lee et al. 2004), production of IFN-γ (Trotta et al. 2008; Bellone et al. 1995; Hunter et al. 1995; Wilson et al. 2011; Meadows et al. 2006), and cytotoxicity (Wilson et al. 2011; Bellone et al. 1995; Rook et al. 1986; Espevik et al. 1988; Kopp et al. 2009). This suppression of NK cell IFN-γ production and cytotoxicity has been suggested as a mechanism of immune invasion in B-acute lymphoblastic leukemia (Rouce et al. 2015). One study has also shown that TGF-β can suppress IFN-γ production *in vivo* (Laouar et al. 2005). There is evidence suggesting that suppression of IFN-γ production after Il-

12 and IL-2 stimulation is not mediated through inhibition of their downstream targets STAT4 and STAT5 (Sudarshan et al. 1999). Increased concentrations of TGF-β in the plasma of cancer patients is also correlated with decreased surface expression of the activating receptor NKG2D (Lee et al. 2004; Crane et al. 2010). In mice infected with lymphocytic choriomenigitis virus (LCMV), TGF-β has also been shown to suppress NK cell proliferation (Su et al. 1993). Developmentally, TGF-β can suppress the maturation of human NK cell precursors (Allan et al. 2010) and in mice, loss of TGF-β signaling on NK cells through a dominant-negative TGF-βRII (dnTGFβRII) results in a huge increase in mature NK cells relative to wild-type mice (Marcoe et al. 2012).

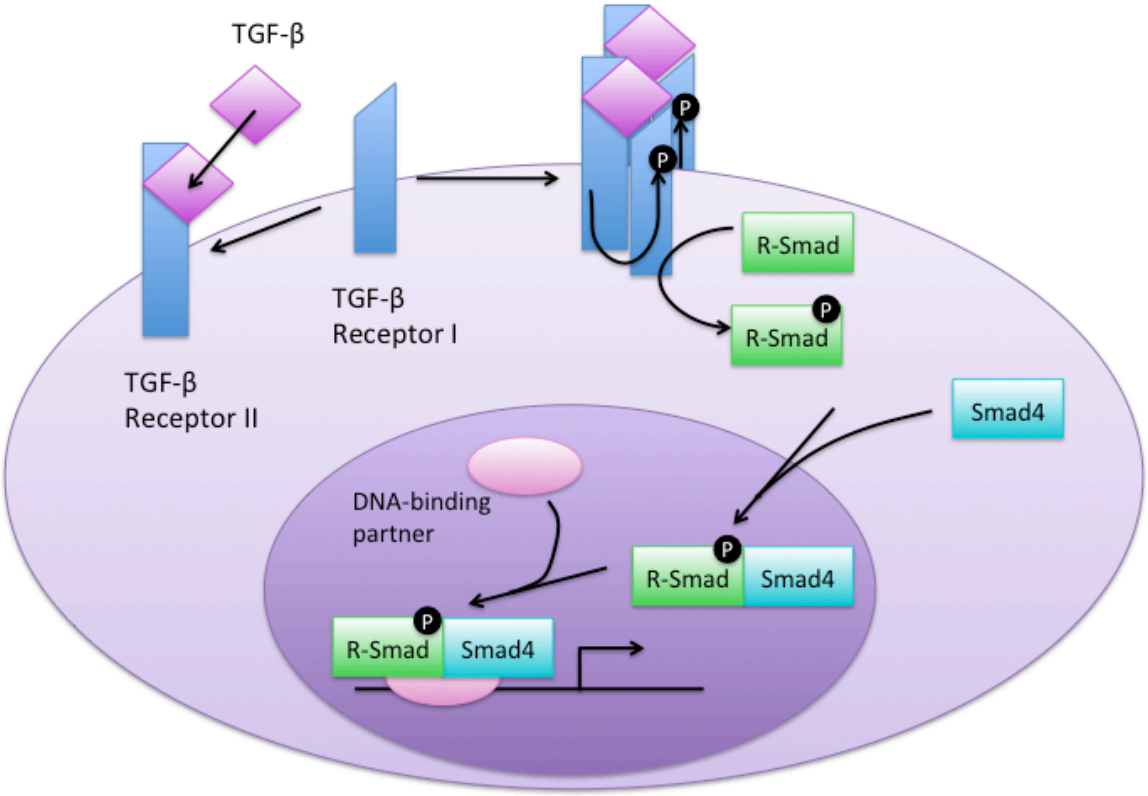


FIGURE 25 | CANONICAL TGF-β SIGNALING PATHWAY

TGF-β binds a TGF-βRII, allowing formation of the tetrameric receptor complex with TGF-βRI. The kinase domain of TGF-βRII then phosphorylates TGF-βRI, allowing an R-Smad to bind and be phosphorylated. This allows the R-Smad to enter the nucleus and bind to a co-Smad, Smad4. Together with a DNA binding partner, this Smad2/3-Smad4 complex is able to modulate transcription of various target genes.

II. Results

1. Generation of *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* Mice

In order to study the role of TGF-β in NK cell development and function, we generated mice with a specific deletion of TGF-β signaling in NK cells by crossing mice carrying a floxed Tgfbr2 (TGF-βRII$^{fl/fl}$) (Chytil et al. 2002) to mice with Cre recombinase knocked-in to the NKp46 gene locus (NKp46^{iCre}) (Narni-Mancinelli et al. 2011). We will hereafter refer to NK cells from these *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice as NK-*TGF-βRII$^{-/-}$* cells or *TGF-βRII$^{-/-}$* NK cells (Figure 26). To confirm specific deletion of the TGF-βRII on NK-*TGF-βRII$^{-/-}$* cells, we first stained for TGF-βRII expression on peripheral NK cells and T cells in *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice (Figure 27A) and compared to Cre-negative littermate controls. As expected, NK-*TGF-βRII$^{-/-}$* cells lacked TGF-βRII expression, while T cells and all cells in littermate controls were unaffected. We also confirmed deletion of TGF-β signaling by testing the ability of TGF-β to suppress production of IFN-γ by NK-*TGF-βRII$^{-/-}$* cells and NK cells from littermate controls. Upon stimulation with IL-12 and IL-18, NK cells from both groups produced IFN-γ (Figure 27B). However, when TGF-β was added to the culture medium, NK cells from littermate mice had diminished IFN-γ production, as expected (Trotta et al. 2008; Wilson et al. 2011; Bellone et al. 1995). NK-*TGF-βRII$^{-/-}$* cells were unaffected (Figure 27B).

2. *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* Mice Have Normal NK Cells at Steady-state

We then investigated the steady state phenotype of NK cells in *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice, as a previous study had shown an increase in mature NK cells in mice expressing a dominant negative TGF-βRII on a CD11c promoter

(Marcoe et al. 2012). Unlike the previous study, however, we see no difference in the frequency of mature NK cells in our *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice compared to their Cre-negative littermate controls (Figure 27C). We also see no difference in various NK cell associated receptors and markers (Figure 27D).

NK-*TGF-βRII$^{-/-}$* cells also appear functionally normal. They produce similar levels of IFN-γ and express similar levels of the degranulation marker Lamp1 (CD107a) in response to *ex vivo* stimulation compared to their littermate controls (Figure 28). They also have no competitive advantage over WT NK cells in reconstituting mixed bone marrow chimeras (Figure 29).

3. TGF-β signaling is Modulated During MCMV Infection

Having seen that NK-*TGF-βRII$^{-/-}$* cells appear normal at steady state, we wanted to investigate whether they would be able to respond normally to MCMV infection. We first tested whether TGF-β signaling might be modulated in NK cells during MCMV infection by performing reverse transcription quantitative real time PCR (RT-qPCR) for TGF-βRII on RNA from sorted NK cells from days 0, 2, 4, and 7 after infection (Figure 30). We found that TGF-βRII expression decreases in NK cells early during infection and increases above resting cell levels by day 7 after infection (Figure 30), indicating that TGF-β signaling may be abrogated early during infection when NK cells are activated. We further looked at phosphorylation of downstream targets of TGF-β signaling, Smad2 and Smad3, by flow cytometry. We found that unlike the TGF-βRII transcript, phospho-Smad2/3 was highest at day 4 after MCMV infection and decreased by day 7 (Figure 31). This could be due to a delay in kinetics, where a downmodulation of

TGF-βRII transcript at day 4 after infection may not affect downstream signals such as Smad2/3 until later.

4. NK-*TGF-βRII*$^{-/-}$ cells Respond Normally to MCMV Infection

To study the behavior of Tgfbr2-deficient NK cells during MCMV infection, we infected mixed bone marrow chimeric mice harboring both WT and *TGF-βRII*$^{-/-}$ NK cells. The mixed chimeric setting best allows us to eliminate influences of different virus dose or degree of inflammation, as both types of NK cells are responding to the same viral load within the same mouse. We found that NK-*TGF-βRII*$^{-/-}$ cells and WT NK cells in this system expressed similar levels of activation markers (Figure 32A), FLICA, and Ki67 (Figure 32B) at day 7 after infection, indicating that NK-*TGF-βRII*$^{-/-}$ cells are phenotypically similar to WT NK cells up to day 7 after MCMV infection.

5. NK-*TGF-βRII*$^{-/-}$ cells Have Defects in Expansion and Persistence in Response to MCMV

To investigate later time points during infection, we employed the adoptive transfer system described previously (Sun et al. 2009). Briefly, we transferred WT and *NKp46*^{iCre} *x TGF-βRII*$^{fl/fl}$ splenocytes from mixed bone marrow chimeras into *Rag2*$^{-/-}$ *x IL-2Rγc*$^{-/-}$ recipient mice, which lack T, B, and NK cells, and infected the recipient mice with MCMV (Figure 32C). This allowed us to track the expansion and contraction of the donor NK cell population in response to MCMV by tracking Ly49H^{+} NK cells. We find that the NK-*TGF-βRII*$^{-/-}$ cells do not expand as well as WT at day 7 after infection, and they appear to have decreased persistence after this initial expansion, shown by their decreased relative

percentage at day 29 after infection compared to day 7 (Figure 32C). However, we again see no difference in activation markers or Ki67 staining between Ly49H^{+} NK-*TGF-βRII*$^{-/-}$ cells and WT NK cells in this setting (Figure 32D).

In CD8^{+} T cells, two distinct effector cell populations are thought to be generated in response to viral infection: KLRG1^{hi} short-lived effector cells (SLECs) and KLRG1^{lo} memory precursor effector cells (MPECs) (Kaech and Wherry 2007). However, we see no difference in KLRG1 expression between our NK-*TGF-βRII*$^{-/-}$ cells and WT NK cells either at steady state or during infection (Figure 27D, 32D). Also in CD8^{+} T cells, the transcription factors T-bet and Eomes are thought to be involved in the MPEC versus SLEC cell fate decision (Figure 33) (Pearce et al. 2003; Intlekofer et al. 2005; Joshi et al. 2007; Hamilton and Jameson 2007; Intlekofer et al. 2007; Banerjee et al. 2010). Therefore, we examined the levels of T-bet and Eomes in NK-*TGF-βRII*$^{-/-}$ cells and WT NK cells in uninfected and infected mixed bone marrow chimeras. We found no significant difference in the levels of T-bet or Eomes expressed at day 0, 2, and 7 after MCMV infection between NK-*TGF-βRII*$^{-/-}$ cells and WT NK cells (Figure 34).

6. NK cells in *IL-10R*$^{-/-}$ Mice Have a Cell-extrinsic Increase in Maturity

Given the relatively mild phenotype we see in our NK-*TGF-βRII*$^{-/-}$ cells, we wondered whether another cytokine might be contributing to suppressing pro-inflammatory signaling during MCMV infection. There is recent evidence that the cytokine IL-10 is expressed during MCMV infection and suppresses NK cell production of IFN-γ and expression of NKG2D and NKp46 (Mandaric et al. 2012). However, that study mainly used an MCMV strain lacking the m157 glycoprotein,

so they did not directly test the role of IL-10 on the Ly49H$^+$ NK cell response to MCMV. Another study used WT MCMV and found that IL-10R blockade resulted in fewer cytotoxic but more apoptotic NK cells in MCMV-infected mice (Stacey et al. 2011), but this study did not address NK cell memory. However, in CD8$^+$ T cells, IL-10 signaling has been shown to be required for the maturation of memory cells after acute LCMV infection (Laidlaw et al. 2015).

We first investigated the steady-state phenotype of NK cells in mice lacking the IL-10 receptor β chain (*IL-10R*$^{-/-}$). We found that *IL-10R*$^{-/-}$ mice had an increase in mature NK cells as measured by CD27 and CD11b and also an increase in NK cells expressing the activation and maturity marker KLRG1 (Figure 35A-B). However, when we generated mixed bone marrow chimeras using *IL-10R*$^{-/-}$ and WT bone marrow, we found that this maturity and activation difference disappeared (Figure 35C-D), indicating that it is cell-extrinsic, either mediated by IL-10R-deficiency in a cell type other than NK cells, or driven by differences in the microbiota between the WT and *IL-10R*$^{-/-}$ mice.

7. NK Cells in *IL-10R*$^{-/-}$ Mice Have a Cell-extrinsic Defect in Persistence After MCMV Infection

We went on to test whether NK cells from *IL-10R*$^{-/-}$ mice had any differences in response to MCMV infection after adoptive transfer. We found that NK cells from naïve *IL-10R*$^{-/-}$ mice had a defect in memory cell generation or persistence after MCMV infection (Figure 36A). This is consistent with a report that KLRG1$^+$ NK cells preferentially expand and generate memory NK cells compared to KLRG1$^-$ NK cells (Kamimura and Lanier 2015), since we had

previously seen that *IL-10R*$^{-/-}$ mice have an increase in KLRG1^{+} NK cells (Figure 35B). Consistently, if we transfer NK cells from *IL-10R*$^{-/-}$ and WT mixed bone marrow chimeras, which have similar levels of KLRG1 expression on both WT and *IL-10R*$^{-/-}$ NK cells, we see no difference in expansion or memory persistence of transferred Ly49H^{+} NK cells (Figure 36B). This indicates that the memory defect in *IL-10R*$^{-/-}$ NK cells is cell-extrinsic and possibly due to their increased maturity at steady-state.

8. DKO NK Cells are More Mature at Steady-state

As we had so far seen relatively weak phenotypes in NK cells lacking either IL-10 or TGF-β signaling alone, we decided to test whether these signaling pathways might operate in a functionally redundant manner. We therefore crossed our two single knockout strains to generate *NKp46*^{iCre} *x TGF-βRII*$^{fl/fl}$ *x IL-10R*$^{-/-}$ (DKO) mice. Given that *IL-10R*$^{-/-}$ NK cells have a cell-extrinsic phenotype, we performed all of our experiments with DKO mice using mixed bone marrow chimeras. We found that DKO NK cells from mixed bone marrow chimeras express normal levels of activation markers (Figure 37A). However, unlike in the *NKp46*^{iCre} *x TGF-βRII*$^{fl/fl}$ or *IL-10R*$^{-/-}$ mice, we observed an increase in mature NK cells among DKO cells compared to WT cells (Figure 37B). In addition, we see a decrease in the expression of Eomes in DKO NK cells compared to WT NK cells at steady-state. This difference is not observed in mixed bone marrow chimeras generated from *NKp46*^{WT} *x TGF-βRII*$^{fl/fl}$ x *IL-10R*$^{+/-}$ littermate controls (Figure 37C). Expression of T-bet was also slightly increased in DKO NK cells, though this difference was not statistically significant (Figure 37C).

9. DKO NK Cells Have a Diminished Response to MCMV

We also tested the levels of T-bet and Eomes in DKO NK cells after MCMV infection of mixed bone marrow chimeras. We found that 7 days after MCMV infection, DKO NK cells express higher levels of T-bet than WT NK cells (Figure 38A). Conversely, DKO NK cells express lower Eomes than WT NK cells, though this difference was not statistically significant (Figure 38B). After adoptive transfer into *Rag2*$^{-/-}$ *x IL-2Rγc*$^{-/-}$ recipients, DKO NK cells do not expand as well as WT NK cells (Figure 38C). These findings are consistent with data in CD8^{+} T cells, where high levels of T-bet are associated with the SLEC fate (Pearce et al. 2003; Intlekofer et al. 2005; Joshi et al. 2007; Hamilton and Jameson 2007; Intlekofer et al. 2007; Banerjee et al. 2010). However, we do not observe any differences in Ki67, Bcl-2, or FLICA staining between transferred DKO and WT NK cells (Figure 38D-E).

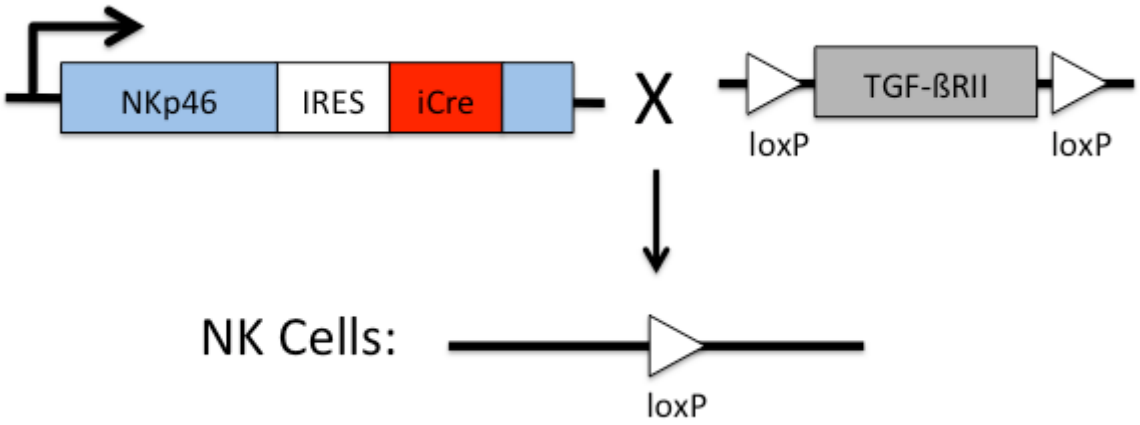


FIGURE 26 | GENERATION OF *NKP46^{iCRE} x TGF-βRII$^{FL/FL}$* MICE

Mice with a specific deletion of TGF-βRII in NK cells were generated by crossing mice carrying Cre recombinase knocked-in to the NKp46 gene locus to mice carrying a floxed TGF-βRII.

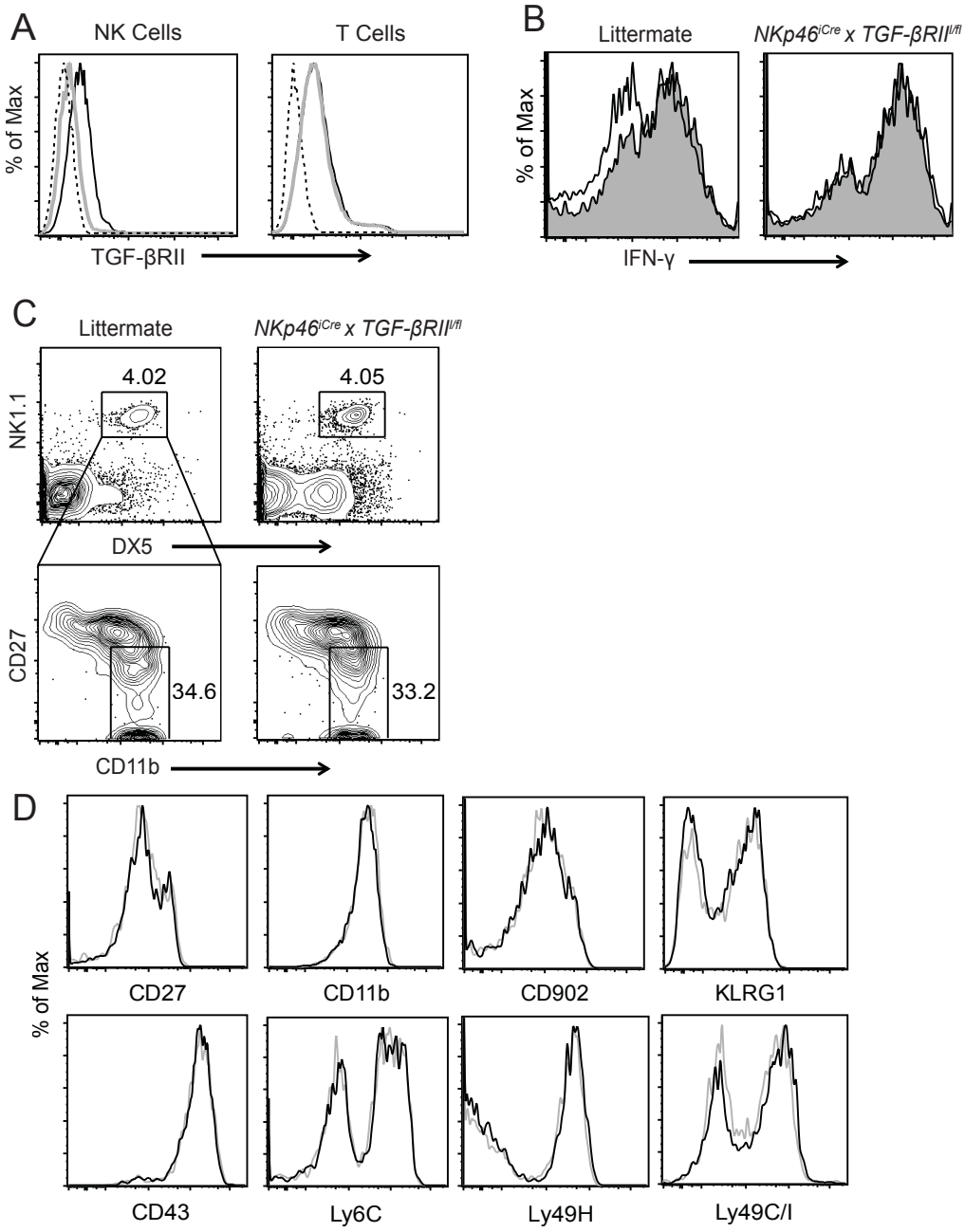


FIGURE 27 | *NKP46^{iCRE} x TGF-βRII$^{FL/FL}$* MICE HAVE NORMAL NK CELLS AT STEADY-STATE

(A) NK cells (left) were confirmed to have deleted TGF-βRII in *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice (gray) compared to Cre^{-} littermates (black). T cells (right) are unaffected. Fluorescence minus one (FMO) control is indicated by the dashed line. (B) NK cells from Cre^{-} littermates (left) and *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice (right) were stimulated with IL-12 and IL-18 with (black) or without (gray shaded) TGF-β. (C) Percentages of total NK cells (top) and mature NK cells within total (bottom) in *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* mice and Cre^{-} littermates are shown by flow cytometry (D) NK cell receptors and activation marker expression on NK-*TGF-βRII$^{-/-}$* cells (gray) and NK cells from Cre^{-} littermates (black). Data are representative of 2-4 experiments with 3-4 mice per group.

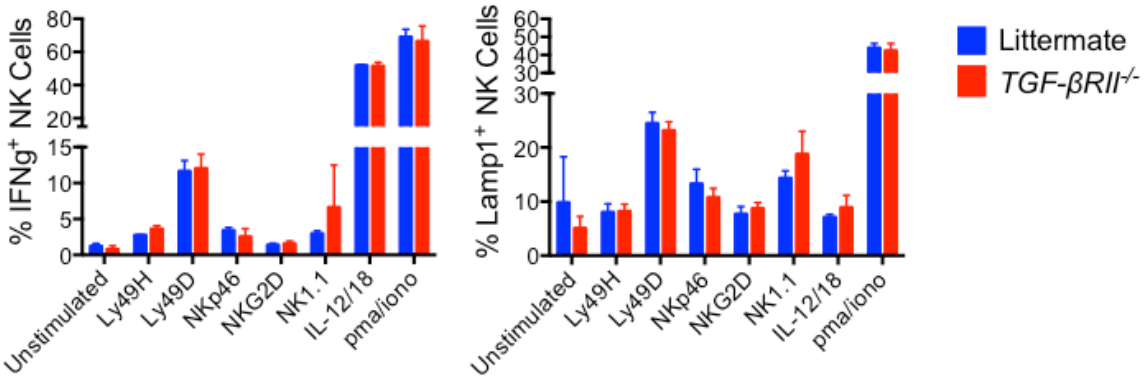


FIGURE 28 | *NKP46^{iCRE} x TGF-βRII$^{FL/FL}$* NK CELLS RESPOND NORMALLY TO *EX VIVO* STIMULATION

NK cells from *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* (red) or Cre^{-} littermate mice (blue) were stimulated with indicated platebound antibodies or cytokines for 5 hours at 37ºC in the presence of golgi plug. The percentage of IFN-γ^{+} (left) or Lamp1^{+} (right) NK cells was measured by flow cytometry. Data are representative of two independent experiments with 2-3 mice per group.

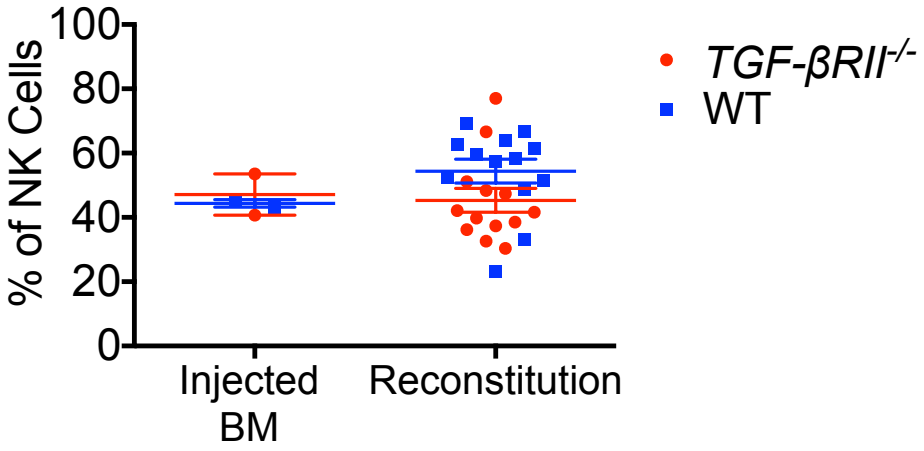


FIGURE 29 | *NKP46iCRE x TGF-βRIIFL/FL* NK CELLS HAVE NO COMPETITIVE ADVANTAGE IN MIXED BONE MARROW CHIMERAS

Pooled data from 2 sets of mixed bone marrow chimeras showing the ratio of injected bone marrow (left) and the reconstitution ratio of NK cells (right) between *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* (red) and WT (blue).

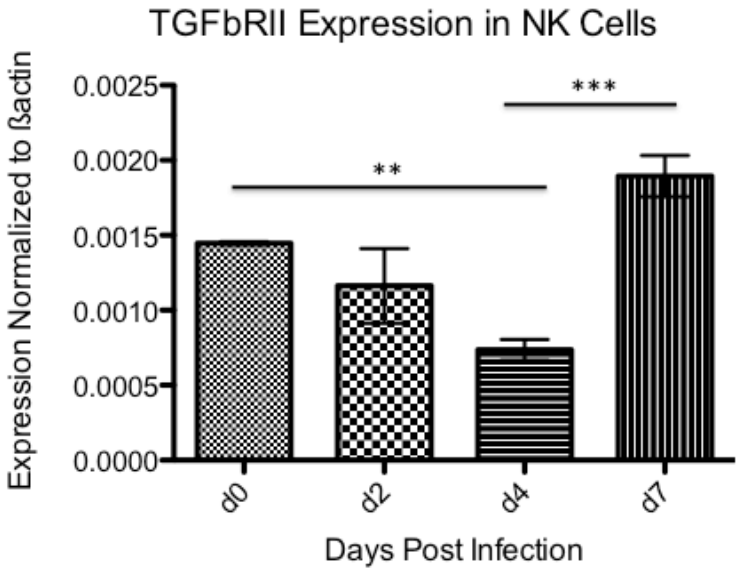


FIGURE 30 | TGF-βRII TRANSCRIPT IN NK CELLS DECREASES EARLY AFTER MCMV INFECTION

RT-qPCR for TGF-βRII was performed on RNA extracted from sorted WT NK cells on indicated days after MCMV infection. N = 2 (d0), 4 (d2,4,7). Differences are not statistically significant unless noted. **, $P \leq 0.01$; ***, $P \leq 0.001$.

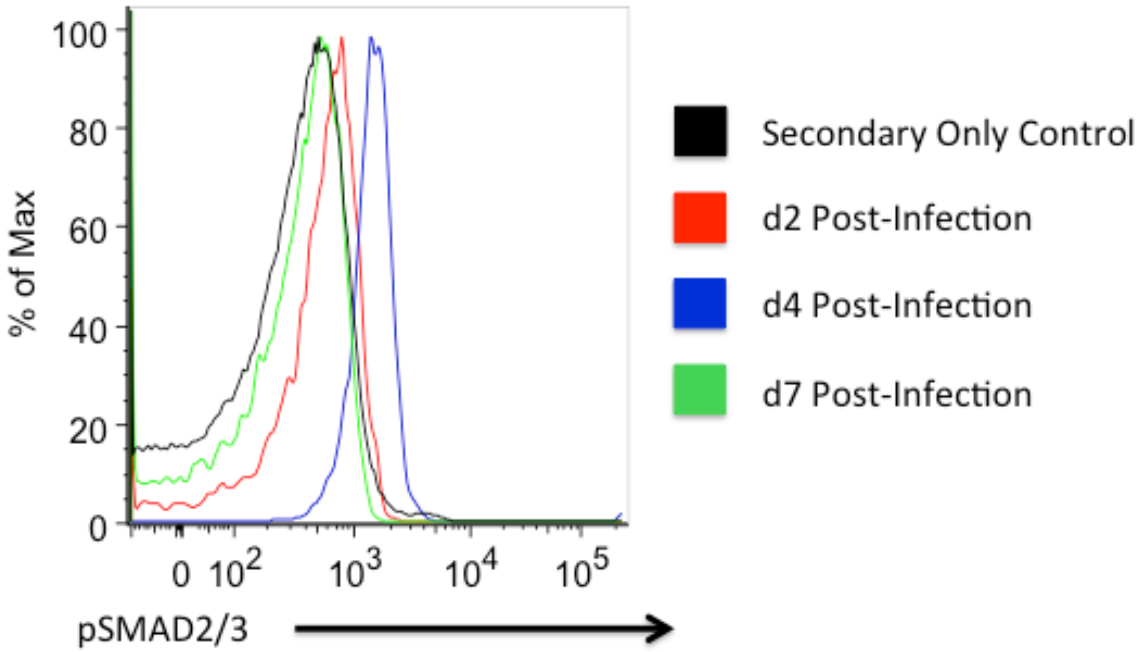


FIGURE 31 | PSMAD2/3 PEAKS AT DAY 4 AFTER MCMV INFECTION

Intranuclear flow cytometry staining was performed on NK cells from WT mice on indicated days after MCMV infection. Gated on $NK1.1^+$ $TCR\text{-}\beta^-$ cells. Data are representative of four mice per group.

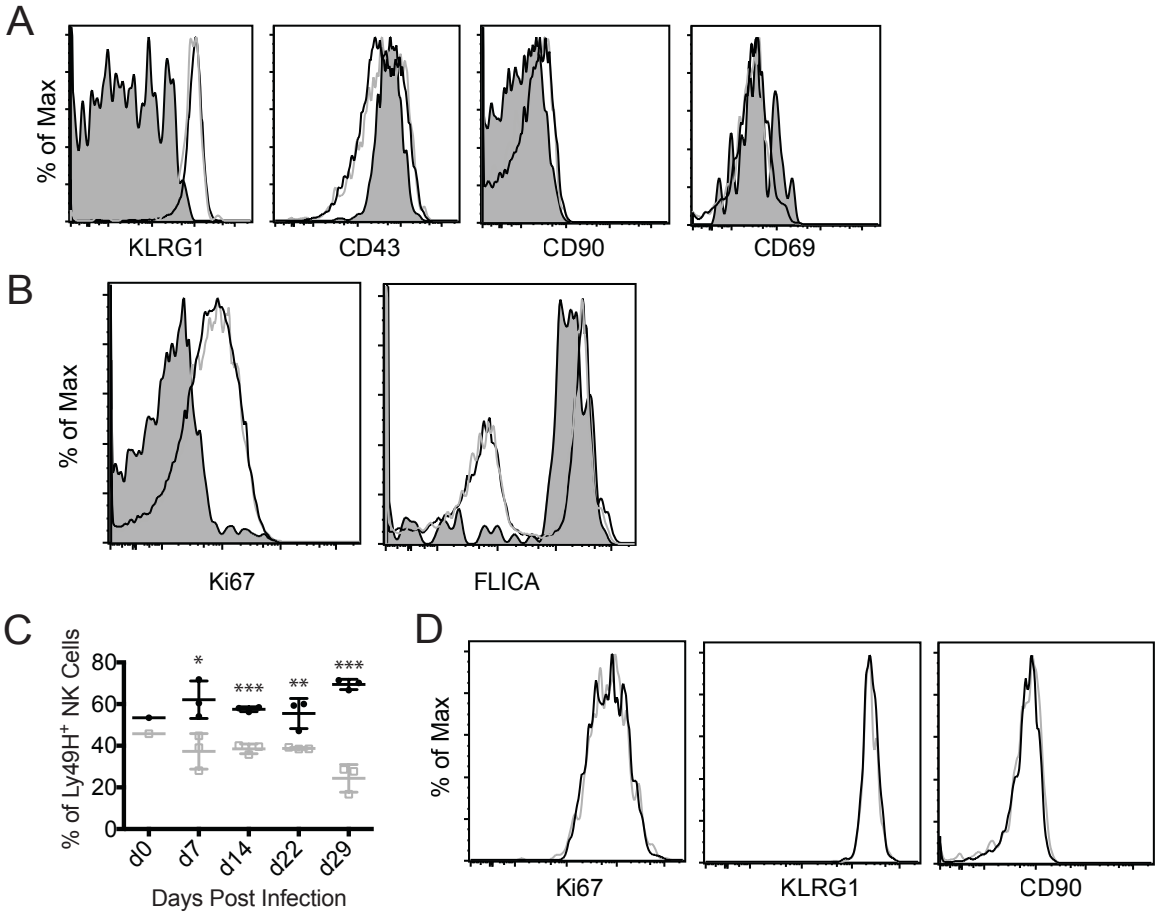
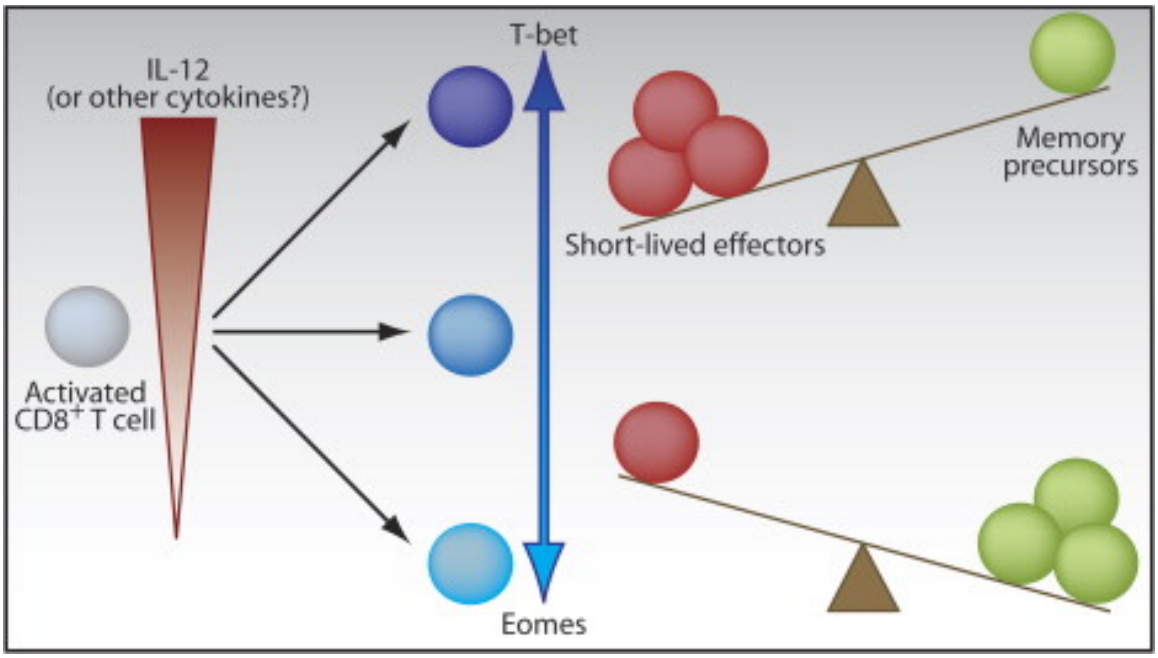


FIGURE 32 | *NKp46^{iCre} x TGF-βRII$^{FL/FL}$* NK CELLS HAVE EXPANSION AND PERSISTENCE DEFECTS AFTER MCMV INFECTION

Activation markers (A) and Ki67, FLICA (B) on *TGF-βRII*$^{-/-}$ (gray), WT (black), and uninfected WT (gray shaded) Ly49H^{+} NK cells in MCMV-infected mixed bone marrow chimeras at day 7 after infection. (C) Relative ratio of *TGF-βRII*$^{-/-}$ (gray) and WT (black) Ly49H^{+} NK cells after adoptive transfer from mixed bone marrow chimeras into *Rag2*$^{-/-}$ *x IL-2Rγ*$^{-/-}$ recipients and MCMV infection. (D) Proliferation and activation markers on Ly49H^{+} *TGF-βRII*$^{-/-}$ (gray) and WT (black) NK cells after adoptive transfer at day 7 after MCMV infection. Data are representative of two (A-B) or four (C-D) independent experiments with 3-4 mice per group. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



Reprinted from Immunity, 27. Hamilton SE, Jameson SC. CD8+ T Cell Differentiation: Choosing a Path through T-bet. 180-182. Copyright (2007), with permission from Elsevier.

FIGURE 33 | T-BET AND EOMES CONTROL CD8$^+$ T CELL MEMORY

Increased levels of T-bet are associated with increased numbers of SLECs (red) over MPECs (green). IL-12 induces T-bet expression and represses Eomes, indicating Eomes may play an opposing role in deciding the SLEC versus MPEC cell fate.

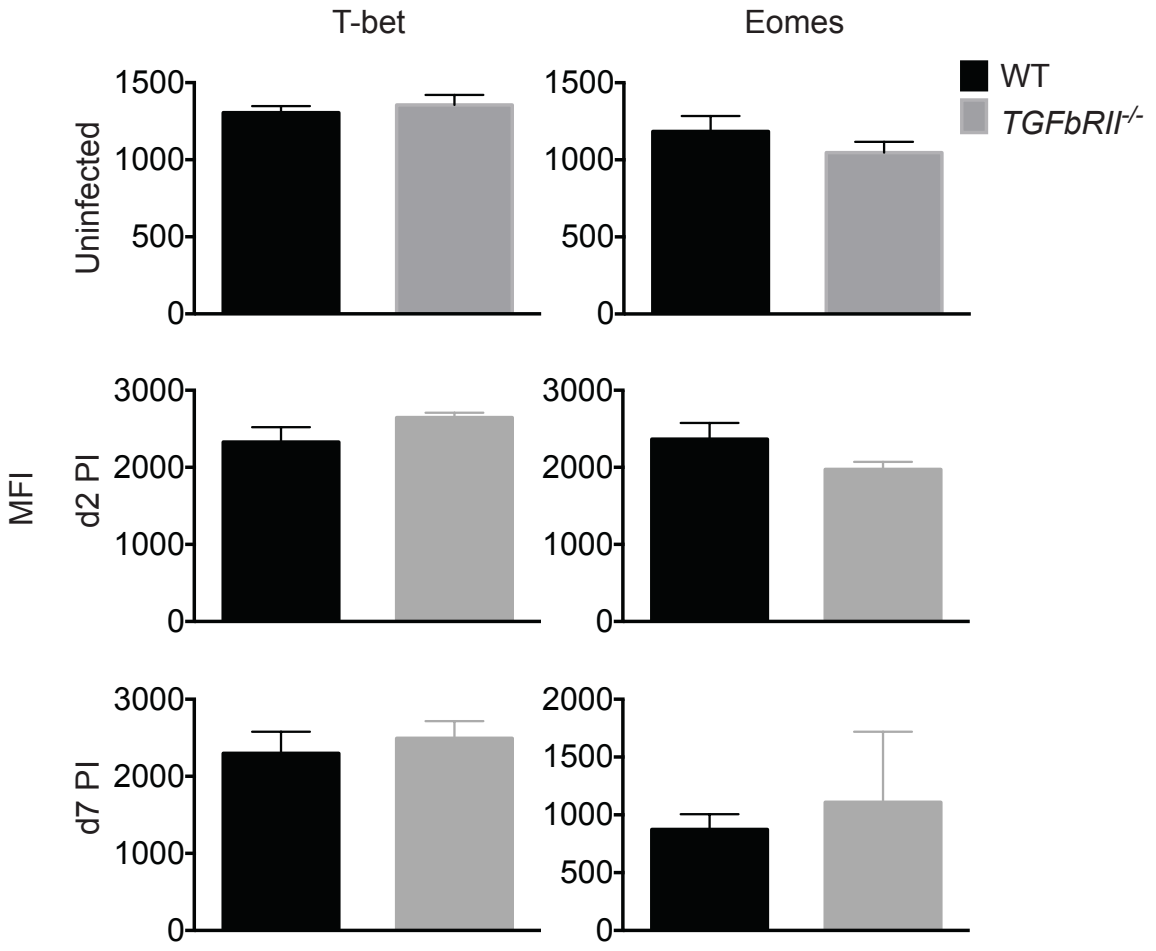


FIGURE 34 | T-BET AND EOMES LEVELS ARE SIMILAR IN *NKP46^{iCRE} x TGF-βRII$^{FL/FL}$* AND WT NK CELLS BEFORE AND AFTER MCMV INFECTION

T-bet and Eomes median fluorescence intensity (MFI) in *TGF-βRII*$^{-/-}$ (gray) and WT (black) NK cells from mixed bone marrow chimeras before (Uninfected) and at days 2 and 7 post infection (PI). Bar graphs show the mean and error bars represent standard deviation (SD) and are representative of two independent experiments with 3-4 mice per group.

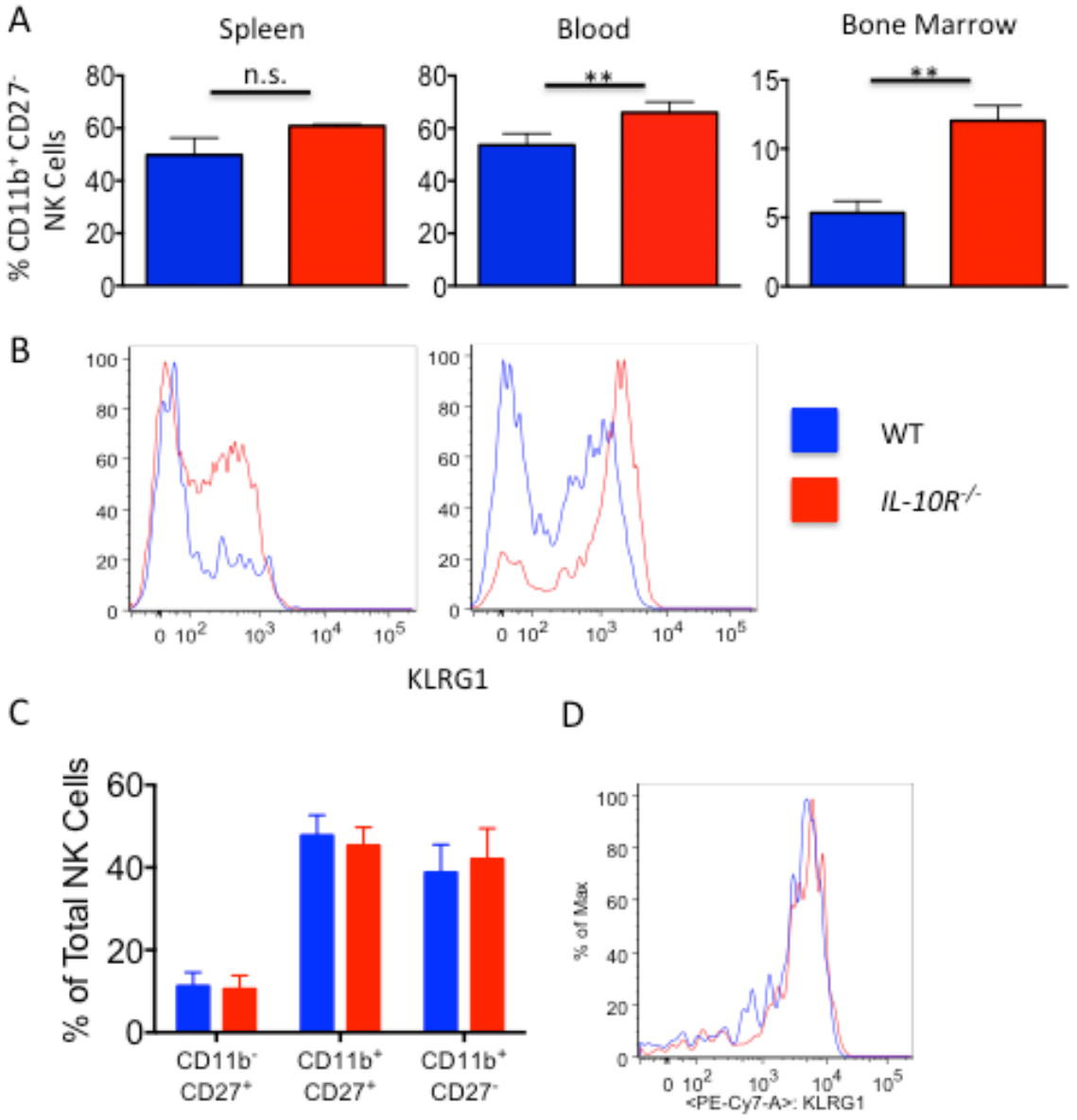


FIGURE 35 | NK CELLS FROM *IL-10R*$^{-/-}$ MICE HAVE A CELL-EXTRINSIC INCREASE IN MATURITY

(A) The percentage of the most mature CD11b^{+}CD27^{-} NK cells was determined by flow cytometry on cell suspensions from various organs. Cells were first gated on TCR-β^{-} NK1.1^{+}. (B) Histograms showing KLRG1 expression on NK cells in spleen (left) and blood (right). N = 3-4 mice per group. (C) Percentage of immature (left), intermediate (center), and mature (right) NK cells in the blood of WT and *IL-10R*$^{-/-}$ mixed bone marrow chimeras. (D) Expression of KLRG1 in blood NK cells from WT and *IL-10R*$^{-/-}$ mixed bone marrow chimeras. Bar graphs represent the mean with error bars showing SD. Data in (C,D) are representative of two independent experiments with 8 mice. Differences are not statistically significant unless noted. **, P ≤ 0.01.

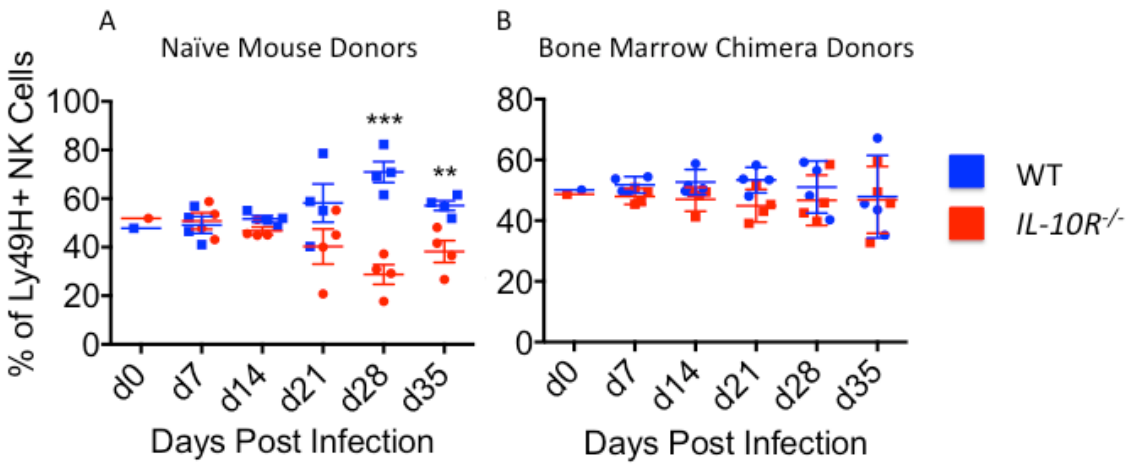


FIGURE 36 | NK CELLS FROM *IL-10R*$^{-/-}$ MICE HAVE A CELL-EXTRINSIC DEFECT IN PERSISTENCE AFTER MCMV INFECTION

(A) The relative ratio of WT (blue) and *IL-10R*$^{-/-}$ (red) Ly49H^{+} NK cells transferred from naïve individual mice into Ly49H-deficient recipient mice and infected with MCMV. (B) The relative ratio of WT (blue) and *IL-10R*$^{-/-}$ (red) Ly49H^{+} NK cells transferred from mixed bone marrow chimeras into Ly49H-deficient recipient mice and infected with MCMV. Data are representative of two independent experiments with 3-4 mice per group. Differences are not statistically significant unless noted. **, $P \leq 0.01$; ***, $P \leq 0.001$.

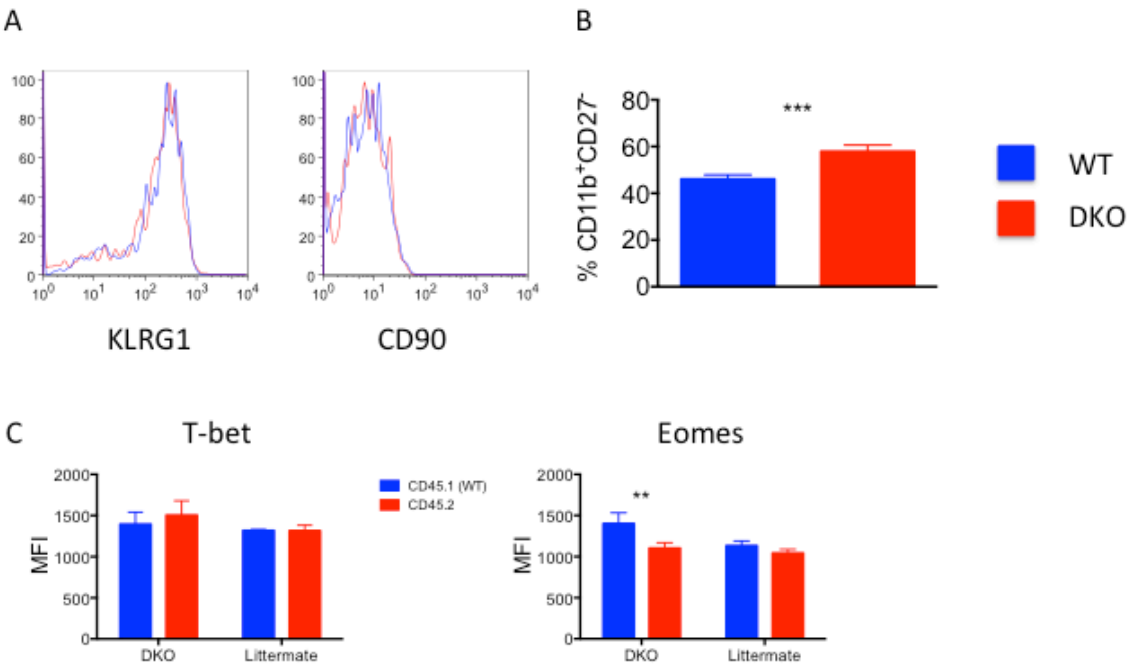


FIGURE 37 | DKO NK CELLS FROM MIXED BONE MARROW CHIMERAS ARE MORE MATURE AND EXPRESS LOWER EOMES THAN WT NK CELLS

(A) Expression of KLRG1 and CD90 on DKO (red) and WT (blue) NK cells from mixed bone marrow chimeras. (B) Percentage of mature NK cells ($CD11b^+$ $CD27^-$) within DKO (red) and WT (blue) NK cells (gated on TCR-β^- $NK1.1^+$) from mixed bone marrow chimeras. (C) Median fluorescence intensity (MFI) of T-bet and Eomes in NK cells from mixed bone marrow chimeras made with WT ($CD45.1^+$, blue) and either DKO or WT littermate ($CD45.2^+$, red) bone marrow. Bar graphs represent the mean with error bars showing SD. Data in (A) are representative of one experiment with 15 mice. Data in (B, C) are representative of two independent experiments with 2-8 mice. Differences are not statistically significant unless noted. **, $P \leq 0.01$; ***, $P \leq 0.001$.

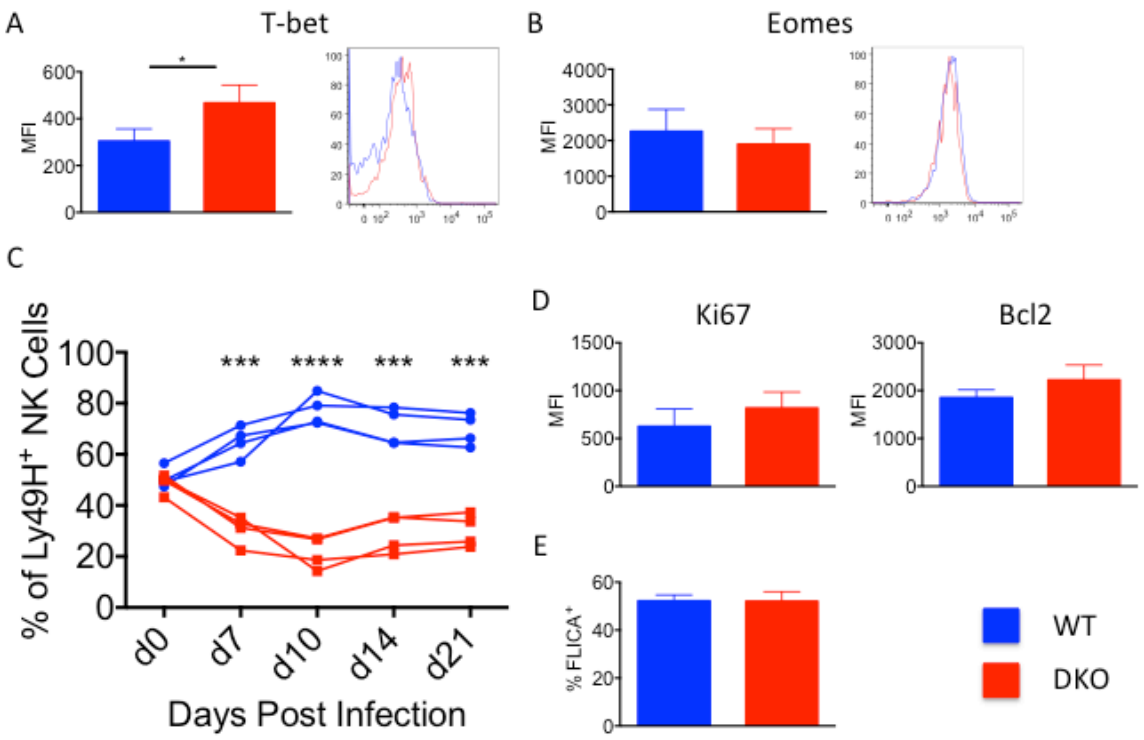


FIGURE 38 | DKO NK CELLS ARE DEFICIENT IN THEIR RESPONSE TO MCMV INFECTION

Median fluorescence intensity (MFI) of T-bet (A) and Eomes (B) in DKO (red) and WT (blue) Ly49H$^+$ NK cells in mixed bone marrow chimeras 7 days after MCMV infection. (C) Relative ratio between DKO (red) and WT (blue) Ly49H$^+$ NK cells from mixed bone marrow chimeras adoptively transferred into *Rag2*$^{-/-}$ *x IL-2Rγ*$^{-/-}$ recipients and infected with MCMV. Connecting lines between time points indicate individual mice. MFI of Ki67 and Bcl-2 (D), and percent FLICA$^+$ (E) in WT (blue) and DKO (red) Ly49H$^+$ NK cells after adoptive transfer and 7 days after MCMV infection. Bar graphs represent the mean with error bars showing SD. Data represent one experiment (A, B, E) or two independent experiments (C, D) with 3-4 mice per group. Differences are not statistically significant unless noted. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001

III. Discussion

Given the importance of TGF-β signaling in other lymphocytes, we were surprised to find no developmental phenotype in our NK-*TGF-βRII*$^{-/-}$ cells. This finding also conflicts with a previously published study using the CD11c-dnTGF-βRII mouse, which showed a huge increase in mature NK cells (Marcoe et al. 2012). One possible reason for this discrepancy is the different promoters used. It is possible that CD11c is expressed earlier than NKp46, allowing deletion of TGF-β signaling at an earlier stage during development and a subsequent maturation phenotype. It is also possible that another CD11c^{+} cell type is mediating the NK cell phenotype in the CD11c-dnTGF-βRII mice. The authors accounted for this by making mixed bone marrow chimeras at different ratios of WT to CD11c-dnTGF-βRII bone marrow, up to 9:1 in favor of WT, and they nevertheless see increased maturity in the CD11c-dnTGF-βRII population (Marcoe et al. 2012). Another possibility is that differences between the dominant-negative and lox/cre systems explain the difference in phenotype. Indeed, it has been shown that the dnTGF-βRII can induce a hyperproliferation of CD8^{+} T cells when expressed on a Cd2 promoter that is not recapitulated by a mouse with a CD4-specific deletion of TGF-βRII (Ishigame et al. 2013). This indicates that the CD8^{+} T cell phenotype may be caused by the dnTGF-βRII construct itself, rather than by absence of TGF-β signaling.

We were similarly surprised to find a lack of obvious phenotype in early viral responses of NK-*TGF-βRII*$^{-/-}$ cells. TGF-β suppresses KLRG1 signaling in resting CD8^{+} T cells *in vitro* and CD8^{+} T cells lacking TGF-β signaling have

increased KLRG1 expression in mice infected with LCMV (Schwartzkopff et al. 2015). However, we saw no differences in KLRG1 expression either at steady-state or during infection in our NK-*TGF-βRII*$^{-/-}$ cells, indicating that KLRG1 may be regulated differently in NK cells. Because the TGF-βRII transcript and Smad2/3 protein data suggest that NK cells do modulate their TGF-β signaling early during MCMV infection, we hypothesized that this cytokine would play an important role in controlling NK cell activation and proliferation. One study had previously shown that NK cells deficient in TGF- β signaling were more adept at controlling MCMV infection (Lewis et al. 2015). However, they used the CD11c-dnTGF-βRII mice, which have more NK cells and more mature NK cells than WT mice (Lewis et al. 2015; Marcoe et al. 2012), complicating the interpretation. It is also possible that MCMV infection simply elicits too strong of a pro-inflammatory environment to see an effect of TGF-β during initial infection. One *in vitro* study has shown that IL-2 and IL-18 can prevent TGF-β-mediated downregulation of NKG2D (Song et al. 2006). As IL-2 and IL-18 are both produced during MCMV infection (Walton et al. 2008; Pien et al. 2000), it is possible that they are able to overcome TGF-β mediated effects. Our observed defect in NK cell expansion and memory generation is consistent with several studies in $CD8^+$ T cells showing a preference towards the SLEC fate in mice with TGF-β insensitive T cells (Sanjabi et al. 2009; Ma and Zhang 2015; Tinoco et al. 2009). In T cells, the MPEC phenotype is associated with increased expression of Eomes, while the SLEC phenotype is associated with increased expression of T-bet (Pearce et al. 2003; Intlekofer et al. 2005; Joshi et al. 2007; Hamilton and Jameson 2007;

Intlekofer et al. 2007; Banerjee et al. 2010). Consistently, T cells lacking TGF-β signaling during infection were shown to have increased T-bet expression and decreased Eomes (Ma and Zhang 2015). Based on this finding, we expected to observe an increase in T-bet and a decrease in Eomes expression in our NK-*TGF-βRII*$^{-/-}$ cells. However, it is possible that we have simply not chosen the optimal timepoint to measure the expression of these molecules to see any differences.

Our data suggest that the TGF-β and IL-10 pathways may cooperate in some fashion in controlling NK cell development and response to MCMV infection, as the DKO NK cells are more mature at steady state and show a greater defect in MCMV responses than NK cells from either single knockout strain. There is some evidence that the pathways do interact, although TGF-β canonically acts through the Smad family of transcription factors, and IL-10 acts through the JAK/STAT pathway with STAT3. STAT3 can be phosphorylated at two different sites, serine 727 and tyrosine 705, which play different roles in controlling embryonic stem cell development (Huang et al. 2014). IL-10 has been reported to induce phosphorylation of both sites in macrophages (Zhu et al. 2015). TGF-β and downstream Smad4 have been shown to suppress tyrosine phosphorylation of STAT3 in cancer cells, leading to a metastatic phenotype (Zhao et al. 2008). Conversely, one group found that a JAK2-specific inhibitor decreased the phosphorylation of STAT3 but also of Smad3 (Liu et al. 2014), indicating that STAT3 signaling may be required for optimal TGF-β signaling in some contexts. In T cells, TGF-β is thought to suppress the negative-feedback

regulator of STAT3, SOCS3, thus enhancing STAT3 activation downstream of IL-6 and driving differentiation of T_h17 cells (Qin et al. 2009). Another study found that Smad2 and Smad3 can act as STAT3 co-activators and co-repressors, respectively, in controlling differentiation of T_h17 cells (Yoon et al. 2015). TGF-β has also been shown to activate STAT3 in human follicular helper T cells (Schmitt et al. 2014). Though these studies were mainly done using IL-6 as the STAT3 activator, it is conceivable that the effects of IL-10, also acting through STAT3, could similarly be augmented or suppressed by TGF-β.

Though TGF-β appears to be dispensable for normal NK cell development, its role in controlling the NK cell effector and memory cell fates is a fascinating line of inquiry. Contrary to our initial expectation, TGF-β signaling appears to be important for optimal NK cell responses against viral infection. This indicates that anti-TGF-β therapies for cancer and other diseases (Akhurst and Hata 2012) may produce the unwanted side effect of inhibiting NK cell control of viral infections. Furthermore, the role of TGF-β signaling in other ILC lineages remains to be investigated. TGF-β has been shown to suppress Gata3 and T-bet in T cells (Gorelik et al. 2000; Heath et al. 2000; Gorelik et al. 2002), leading to the question of whether it plays a role in suppressing these factors during ILC development. Recent evidence further suggests that TGF-β promotes development of lung ILC2 (Denney et al. 2015), supporting the importance of investigating TGF-β in ILC development and function.

IV. Materials and Methods

1. Mice

WT C57BL/6, congenic (CD45.1$^+$ and CD45.1$^+$ x CD45.2$^+$), *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* (Narni-Mancinelli et al. 2011; Chytil et al. 2002), *IL-10Rβ$^{-/-}$* (Jackson Laboratory), *NKp46^{iCre} x TGF-βRII$^{fl/fl}$* x *IL-10R$^{-/-}$* mice were bred at MSKCC in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). All experiments were conducted in accordance with IACUC and MSKCC approval and guidelines. Mixed bone marrow chimeric mice were generated as previously described (Sun et al. 2009). WT controls were age and sex matched C57BL/6 mice or WT littermates as indicated.

2. Viral Infections

MCMV (Smith strain) was obtained from L. Lanier (University of California San Francisco). MCMV was twice passaged through BALB/c hosts and stocks were generated by douncing salivary glands of mice 3 weeks after MCMV infection.

Mixed bone marrow chimeric mice were infected by i.p. injection of approximately 7.5×10^3 PFU of MCMV in 500 uL of PBS. For the adoptive transfer studies, mice were infected by i.p. injection of approximately 7.5×10^2 PFU of MCMV in 500 uL of PBS one day after receiving approximately 1×10^5 Ly49H$^+$ NK cells by intravenous (i.v.) injection.

3. Flow Cytometry and Cell Sorting

Single cell suspensions from the indicated organs were stained in media or PBS with the anti-Fc receptor antibody 2.4G2 and indicated monoclonal antibodies

(Tonbo, Biolegend, eBioscience, BD Biosciences) for 20 minutes on ice for surface receptors. Intracellular and intranuclear staining was performed by fixing and permeabilizing with the Foxp3/Transcription Factor Staining Kit (eBioscience) according to manufacturer instructions. For TGF-βRII staining, cells were first incubated with biotinylated TGF-βRII polyclonal antibody (R&D Systems), followed by incubation with streptavidin conjugated to the PE-Cy7 fluorophore. Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively using FACSDiva software (BD Biosciences). For experiments involving qRT-PCR, cell populations were sorted to >95% purity. Data were analyzed with FlowJo 9.8.5 software (Tree Star).

4. *Ex Vivo* Stimulation of NK cells

In 96-well plates, approximately 1 x 10^6 splenocytes were stimulated for 5 h in RPMI containing 10% FBS and a 1:1000 dilution of golgi plug (BD Biosciences) with recombinant mouse IL-12 (20 ng/ml; R&D Systems), IL-18 (10 ng/ml; R&D Systems), TGF-β (10 ng/mL; R&D Systems), PMA (50 ng/mL), ionomycin (1 ug/mL), or with plate-bound antibodies (10 μg/ml; eBioscience) against the activating NK cell receptors Ly49H, Ly49D, NKp46, NKG2D, or NK1.1 as indicated.

5. Quantitative Real-time PCR

NK cells were sorted to >95% purity from total splenocytes using an Aria II sorter (BD Biosciences), and lysed in Tri-Reagent (Ambion). RNA purification was accomplished using the RNeasy kit (with on-column DNase I treatment; QIAGEN), and cDNA synthesis was carried out using MuLV reverse transcription

and Oligo $dT_{(16)}$ primers (Applied Biosystems). iQ Sybr Green SuperMix (Bio-Rad Laboratories) was used for qRT-PCR. Data were normalized to β-actin and expressed as relative target abundance via the ΔΔCT method, where Ct (threshold cycle) is the cycle number at which the amplification curve intersects the threshold value. Relevant primer sequences are as follows: TGF-βRII forward, 5′-CCGCTGCATATCGTCCTGTG-3′; TGF-βRII reverse, 5′-AGTGGATGGATGGTCCTATTACA -3′; β-actin forward, 5′-TGCGTGACATCAAAGAGAAG-3′; and β-actin reverse, 5′-CGGATGTCAACGTCACACTT-3′.

6. Statistical Analysis

Bar graphs represent mean with error bars showing ± SD unless otherwise indicated. Data were analyzed using two-tailed unpaired Students t-test with Welch's correction or one-way ANOVA (with correction for multiple comparisons where applicable). All analyses were performed using Prism 6.0 (GraphPad) software, and differences were considered significant when $p \leq 0.05$. Asterisks in figures denote statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Differences are not significant unless noted.

Chapter 4: Discussion

I. Evolution of ILCs

Although the importance of ILCs in immunity and homeostasis is evident (Figure 9), it is interesting to consider how and why these “innate versions” of T cells came into existence. T cells as we know them, expressing antigen receptors generated by V(D)J recombination mediated by the RAG recombinase, first appeared in jawed vertebrates 450-600 million years ago (Kasahara et al. 2004). In jawless fish, such as the lamprey and hagfish, lymphocyte-like cells express variable lymphocyte receptors (VLR), which are generated by somatic recombination that is independent of RAG recombinase and Activation-induced cytidine deaminase (AID) (Pancer et al. 2005; Kang and Malhotra 2015). The discovery of two related but distinct systems of gene rearrangement (V(D)J and VLR) suggests that adaptive immune cells may have evolved from a common ancestor, potentially an ILC (Walker et al. 2013; Eberl et al. 2015). This idea is supported by the evolutionary history of transcription factor networks, with ILC-promoting transcription factors Gata3, T-bet, Id2, and PLZF emerging before the RAG recombinase (Kang and Malhotra 2015). ILCs also develop earlier than adaptive lymphocytes during mammalian ontogeny (Eberl 2012), suggesting their importance in early protection of neonates against pathogens. This also suggests that ILCs may have evolved earlier than adaptive lymphocytes, as organisms lacking early immunity provided by ILCs would have a higher likelihood of dying before reproductive age.

However, the opposite argument could also be made: if adaptive lymphocytes evolved first, the emergence of ILCs would have conferred a survival advantage resulting in all later organisms containing both lineages. In addition, lymph nodes appear to have evolved rather late, possibly as late as reptiles and birds (Boehm et al. 2012), indicating that LTi cells either evolved later than adaptive lymphocytes, or that they fulfilled a different function in lower organisms. Future studies will be needed to identify whether ILCs can be found in lower organisms.

Of particular interest is whether Nfil3 is expressed in non-vertebrates. Nfil3 has been identified in zebrafish, salmon, and grass carp (H. Yu et al. 2014), and data from Ensembl suggests that Nfil3 homologs exist as early as arthropods and nematodes (Yates et al. 2016) (Figure 39). However, as Nfil3 has many different functions in mice, it remains to be investigated whether it fulfills the same ILC-promoting role in other organisms. Regardless of the evolutionary history of ILCs, it seems clear that the fast-acting nature of ILCs remains desirable, even in the presence of adaptive immunity. In *Citrobacter rodentium* infections in particular, both early ILC-expressed and later T cell-expressed IL-22 is necessary for full protection (Basu et al. 2012). In addition, NK cells are necessary for control of human viruses, even in the presence of B and T cells (Orange 2006; Biron et al. 1989; Etzioni et al. 2005). The critical nature of ILCs is further supported by our own data showing that *Nfil3*$^{-/-}$ mice, which lack innate lymphoctyes but have largely intact adaptive immunity, are nevertheless susceptible to intestinal pathogens.

II. Nfil3-Independent ILCs

Understanding the signals controlling ILC development and function will help us to further elucidate how ILCs fit in the greater context of pathogen and tumor immunity. Here, we have shown that the transcription factor Nfil3 is a critical regulator of ILC development, necessary for the transition from the CLP to the αLP ILC precursor. The factors controlling Nfil3 expression are largely unknown, though it is thought to be induced by IL-7 and IL-15 in ILC progenitors and NK cells, respectively (Xu et al. 2015; Gascoyne et al. 2009; M. Yang et al. 2015). Further work is necessary to more fully understand the many signals upstream and downstream of Nfil3.

One critical outstanding question is how some residual ILCs are able to develop in Nfil3-deficient mice. Although the magnitude of ILC deficiency varies between organs, most show at least a small percentage of ILCs. This deficiency is more pronounced in mixed bone marrow chimeras, but is nevertheless incomplete. One possibility is that different cytokine milieu in different organs is able to induce a tiny percentage of ILC precursors to escape the requirement for Nfil3, and that in the absence of any other ILCs with which to compete, this tiny percentage is amplified, though not to WT levels. This idea is supported by the finding that Nfil3-deficient bone marrow produces smaller percentages of ILCs in mixed bone marrow chimeras compared to full knockout mice, as Nfil3-deficient ILCs in these mixed chimeras must compete with WT ILCs for cytokines and other signals. This idea is also supported by the finding that Nfil3-independent

NK cells can be driven out by activating receptor engagement or bystander proinflammatory cytokines following viral infection (Firth et al. 2013).

Furthermore, it is possible that these Nfil3-independent ILCs are able to develop through the actions of another bZIP or PAR family transcription factor. These factors have similar binding sites to Nfil3 (Cowell 2002), and if they are able to bind Nfil3 sites at low efficiency, this may account for small numbers of ILCs being able to develop independently of Nfil3. Another possibility is that Nfil3-independent ILCs are derived from fetal precursors and that these precursors are themselves Nfil3-independent. Nfil3-deficient fetal livers have been shown to contain more CHILP cells than adult bone marrow (Xu et al. 2015). The idea of separate fetal and adult ILC precursors is further supported by data in AhR-deficient mice, which have intact early-develpoping lymphoid structures such as lymph nodes and Peyer's patches—suggesting intact fetal LTi cells—but lack later developing cryptopatches and lymphoid follicles, suggesting later LTi deficiencies (Lee et al. 2012; Kiss et al. 2011; Qiu et al. 2012). In addition, an ILC precursor has been identified in the fetal intestine that may be responsible for early seeding of ILCs in that organ (Bando et al. 2015). It is currently unknown whether this precursor depends on Nfil3.

Our finding that residual ILCs that develop in *Nfil3*$^{-/-}$ mice are deficient in cytokine production could support either a cytokine-driven "escape" model or a separate (possibly fetal) origin model for the development of Nfil3-independent ILCs. In the former case, Nfil3-independent ILCs are perhaps overstimulated and/or exhausted due to their lack of competition for cytokines and other

activating signals and are therefore somewhat refractory to further stimulation. In the latter case, Nfil3-independent ILCs may represent a separate but related ILC lineage with different stimulation requirements or function. Different tissue and developmental stage-specific requirements for ILC precursors would also help explain the conundrum of NK cells and non-NK cell ILC1.

III. The Question of ILC1s

There is still great confusion and controversy in the field on the lineage relationship between conventional NK cells and non-NK cell ILC1. Though NK cells and ILC1 both express the transcription factor T-bet and produce IFN-γ (Klose et al. 2014; Bernink et al. 2013; Fuchs et al. 2013; Seillet et al. 2015), NK cells are now thought to be a separate lineage from “helper” ILCs, which develop from the CHILP (Klose et al. 2014). This notion is supported by transcriptional profiling showing that while NK cells and ILC1 are related by principal component analysis, they do cluster separately, and genes upregulated in ILC1 over NK cells are also upregulated in other ILCs, whereas genes upregulated in NK cells are generally not expressed in other ILCs (Robinette et al. 2015). Nevertheless, NK cells and ILC1 share some surface markers, including the activating receptors NK1.1 and NKp46, but can most easily be distinguished by their differential expression of CD49b (NK cells), or CD49a and TRAIL (ILC1) (Seillet et al. 2015) (Figure 40). Additionally, ILC1 and NK cells both express lytic molecules such as perforin and granzymes (Daussy et al. 2014), though NK cells are thought to express higher levels except in the liver (Robinette et al. 2015). Both NK cells and ILC1 can therefore exhibit killing activity, though ILC1 may kill through

different mechanisms, such as TRAIL receptor engagement (Kiessling, Klein, Pross, et al. 1975; D K Sojka et al. 2014; Daussy et al. 2014; Dadi et al. 2016).

Various precursors able to develop into all ILCs, including NK cells, have been identified and been shown to require Nfil3, Tcf-1, and Tox (Q. Yang et al. 2015; X. Yu et al. 2014; Seehus et al. 2015). These data indicate that NK cells and ILC1 do share an early lineage relationship. Conversely, ILC1 in the skin, uterus, salivary glands, liver, and thymus have been shown to develop in Nfil3-deficient mice (D K Sojka et al. 2014; Cortez et al. 2014; Seillet, Rankin, et al. 2014), casting doubt on whether these cells are truly derived from the same Nfil3-dependent precursor as NK cells. However, our data suggests that ILC1 found in the mesenteric lymph node, small intestine, liver, and adipose tissue are absolutely dependent on Nfil3, particularly evident in mixed bone marrow chimera studies (Figure 11 and O'Sullivan, T. unpublished observations), consistent with our observation that Nfil3-deficient mice lack an early ILC precursor (Geiger et al. 2014). Furthermore, one study found that intraepithelial ILC1 were severely reduced in Nfil3-deficient mice and that these ILC1 show TGF-β imprinting evidenced by CD103 expression (Fuchs et al. 2013). However, we have not seen any dependence or effect of TGF-β on conventional NK cell development.

Interestingly, intraepithelial ILC1, unlike ILC1 from other tissue sites, have been shown to express transcripts associated with both conventional NK cells and ILC1, making their classification difficult (Robinette et al. 2015). These conflicting observations leave the relationship between different group 1 ILC

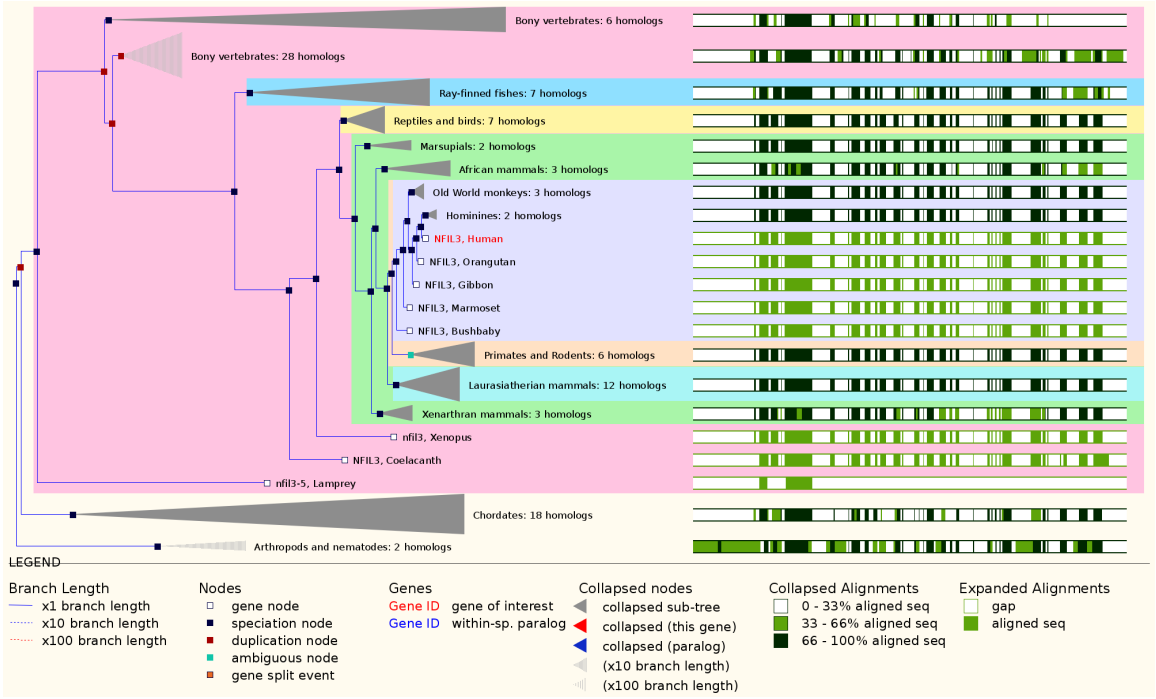
subsets and early Nfil3-dependent ILC precursors somewhat unclear. However, as discussed earlier, it may be that different tissue-specific or microbiota-dependent signals are able to bypass the requirement for Nfil3 at low efficiency, accounting for the differing observations of Nfil3-independent ILC1.

IV. Summary

We have shown that the transcription factor Nfil3 is an early regulator of ILC development, including NK cells and LTi cells, highlighting the lineage relationships between these cells and other ILC subsets. The lack of all ILC subsets additionally makes the *Nfil3*$^{-/-}$ mice a useful tool to study the importance of ILCs in disease models in the presence of adaptive immunity. We have shown that *Nfil3*$^{-/-}$ mice are more susceptible to *C. rodentium* and *C. difficile* infection. However, they do not show major differences in colitis compared to WT mice after DSS-treatment, indicating that ILCs may not be the major players preventing pathology in that setting. In addition, *Nfil3*$^{-/-}$ mice are less susceptible to AOM/DSS induced colon tumors, suggesting that ILCs can be tumor promoting in certain contexts. Indeed, IL-22 has been previously shown to be tumor-promoting in mouse and human colon cancer (Kirchberger et al. 2013; Jiang et al. 2013), making it a promising clinical target (Sabat et al. 2013). Future studies will investigate which ILC subsets are tumor promoting and which mediate anti-tumor immunity, and how these cells might interact with adaptive lymphocytes in the pathogenesis of colon cancer.

Among ILCs, NK cells, as the founding member, are the best characterized and understood. Their role in immunity to pathogens and tumors is

well documented and they are generating increasing interest as a target for immune therapies (Vivier et al. 2012; Dahlberg et al. 2015). Therefore, understanding the signals controlling NK cell development and function will be critical for future therapeutic innovations. Here, we have shown that the critical immunoregulatory cytokine TGF-β is largely dispensable for normal NK cell development, though it may be involved in the development of ILC1 (Fuchs et al. 2013). Understanding these important signals that govern ILC development will be fundamental in future studies of how these cells are able to mediate both disease protection and pathogenesis.



Herrero, J. Muffato, M. Beal, K. et al. Ensembl comparative genomics resources. *Database (Oxford)* 2016 pii:bav096. PubMed PMID: 26896847; PubMed Central PMCID: PMC4761110 doi:10.1093/database/bav096.

FIGURE 39 | NFIL3 EXPRESSION DURING EVOLUTION

Homologs to human Nfil3 exist among various invertebrates and vertebrates.

Tissue Locations	All	liver, thymus, intestine, skin, uterus, salivary glands
Surface Markers	NK1.1, NKp46, CD49b	NK1.1, NKp46, CD49a, TRAIL, IL-7R
Killing	Yes	Yes
IFN-γ production	Yes	Yes
Tissue Resident	No	Yes
T-bet Dependent	Yes	Yes
Eomes Dependent	Yes	No
Nfil3 Dependent	Yes	No?

FIGURE 40 | NK CELLS AND ILC1

Differences between conventional NK cells (left) and non-NK cell ILC1 (right) as currently understood.

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