DEVELOPMENT AND HETEROGENEITY OF TYPE 2 INNATE LYMPHOID CELLS IN MOUSE LUNGS

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Abstract

Lung group 2 innate lymphoid cells (ILC2s) drive allergic inflammation and promote tissue repair. To investigate the early developmental pathways that leads to the generation of lung ILC2, I divided adult bone marrow lymphoid primed multipotent progenitor (LMPPs) into $CD127^{-}$ (LMPP-s) and $CD127^{+}$ (LMPP+s) subsets and compared them with $Ly6D^{-}$ and $Ly6D^{+}$ common lymphoid progenitors (CLPs). Although, all lymphocytes are thought to develop from CLPs, LMPP+s differentiated into T cells and ILCs more rapidly and efficiently than other progenitors in transplantation assays. These results suggested that some ILCs and T cells may develop from LMPP+s via CLP-independent pathways. To investigate whether distinct ILC2 subsets mediate the distinct ILC2 functions and elucidate their developmental relationship, we generated RORα lineage tracer mice and analyzed them by single cell RNA sequencing, flow cytometry and functional assays. Adult lung ILC2s were divided into IL-$18R\alpha^{+}ST2^{-}$ and IL-$18R\alpha^{-}ST2^{+}$ subsets. The former had an immature ILC phenotype, produced little cytokines and contained ILC progenitor like cells expressing *Tcf7*, whereas the latter was conventional ILC2s. Neonatal lung conventional ILC2s (IL-$18R\alpha^{-}ST2^{+}$) were divided into two distinct effector subsets and an IL-$18R\alpha^{+}ST2^{-}$*Tcf7*$^{+}$ progenitor-like subset. The two effector subsets were defined by the expression of ICOS and KLRG1, and they differentially produced the growth-factor amphiregulin and type 2 cytokines. Therefore, effector ILC2s diverge into tissue-repairing and pro-inflammatory subsets, which differ in transcriptional and phenotypic properties. The IL-$18R\alpha^{+}ST2^{-}$*Tcf7*$^{+}$ cells are likely IL-18 responsive lung ILC progenitors, which may contribute to ILC-poiesis in neonatal and inflamed lungs.

Lay Summary

Our laboratory has characterized the functions of a lymphocyte population called ILC2 in mouse lungs. ILC2s contribute to the development of significant respiratory diseases including allergy, asthma and fibrosis. My doctoral research has elucidated the developmental pathways and complexity of lung ILC2s. I have showed that ILC2 development does not follow the conventional pathway of lymphocyte development. I have also showed that more restricted progenitors for ILC2s seem to seed neonatal and adult mouse lungs and generate ILC2s inside the lung, which might have implications for therapies targeting ILC2s. Plus, I have found that upon activation, ILC2s develop into two subsets, one that promotes inflammation (pro-inflammatory) and one that mediates tissue repair (tissue-repairing). I believe that pro-inflammatory ILC2s should be targeted in allergy and asthma, whereas tissue-repairing ILC2s are needed for the repair of the damaged tissue. On the other hand, tissue-repairing ILC2s could be targeted in cystic fibrosis.

Preface

A version of Chapter 3 has been published. [Ghaedi, M], Steer, C. Martinez-Gonzalez, I. Halim, T. Abraham, N. and Takei, F. (4/2016) Common lymphoid progenitor-independent pathways of innate and T lymphocyte development. Cell Reports. 15(3): 471-480. I designed and performed the experiments, wrote and reviewed the paper. Martinez-Gonzalez, I., Steer, C., and Halim, T. performed experiments and reviewed the paper (the experiments depicted in Figure 3.5A were solely done by Martinez-Gonzalez, I.). Abraham, N. supervised the project, designed the experiments. Takei, F. supervised the project, designed the experiments and wrote the paper.

A version of chapters 4 and 5 are under revision. [Ghaedi, M], Shen, ZY. Martinez-Gonzalez, I. Wei, L. Lu, X. Heravi-Moussavi, A. Marra, M. Bhandoola, A. and Takei, F. (12/2018) Single cell analysis of RORα lineage tracer mice revealed functional heterogeneity of lung ILC2s. I designed, performed the experiments, analyzed data and wrote the paper. Shen, ZY. designed, performed experiments and bioinformatics analyses of single cell RNA-sequencing data. Wei, L. and Heravi-Moussavi, A. performed bioinformatics analyses single cell RNA-sequencing data. Martinez-Gonzalez, I. performed experiments. Lu, X. designed and performed experiments in Figure 4.7 and Figure 5.6A. Marra, M. supervised the bioinformatics analysis. Bhandoola, A. designed and supervised the research. Takei, F. designed and supervised the research and wrote the paper.

All animal use was approved and performed in accordance with the guidelines of the animal care committee of the University of British Columbia and the Canadian Council on Animal Care. Canadian Council on Animal Care approval was granted under the certificate numbers: A16-0131 and A18-0231.

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List of Abbreviations

AB: antibody

AP1: activator protein 1

APC: allophycocyanin

B6: C57Bl/6

BAFF: B-Cell-Activating Factor

BCL11b: B cell leukemia/lymphoma 11B

BM: bone marrow

CHILPs: common progenitor to all helper-like ILCs

CLP: common lymphoid progenitor

CMP: common myeloid progenitors

COPD: chronic obstructive pulmonary disease

CRTH2: chemoattractant receptor-homologous molecule expressed on TH2 cells

DCs: dendritic cells

EGFP: enhanced green fluorescent protein

EILPs: early ILC progenitors

ETPs: early thymic progenitors

ETS: E26 transformation specific

FACS: florescence activated cell sorting

FOXP3: forkhead box P3

GATA3: GATA-binding protein 3

GFI-1: growth factor independent 1

GFLs: glial cell-derived neurotrophic factor family of ligands

HLH: Helix-Loop-Helix

HMG: high mobility group

HSC: haematopoietic stem cell

ID2: Inhibitor of DNA-binding 2

IFN: interferon

IFNγ: interferon gamma

IL-17RB: IL-25 receptor

IL: interleukin

ILC: innate lymphoid cell

ILC1: innate lymphoid cell group 1

ILC2: innate lymphoid cell group 2

ILC2Ps: ILC2 progenitors

ILC3: innate lymphoid cell group 3

ILCPs: ILC precursor

ILFs: isolated lymphoid follicles

IRF: interferon-regulatory factor

Lin: lineage

LMPP: lymphoid primed multipotent progenitor

LN: lymph node

LT: lymphotoxin

LTi: lymphoid tissue inducer

Ly49: lymphocyte antigen 49 complex

MHC: major histocompatibility complex

NFIL3: nuclear factor interleukin-3

NK: natural killer

NKT: natural killer T cell

NOD/SCID: non obese diabetic/severe combined immunodeficient

NSG: NOD/SCID IL-2 receptor gamma chain knockout

PE: phycoerythrobilin

RAG: recombinase activating gene

ROR: retinoic acid receptor related orphan nuclear receptor

Sca-1: stem cell antigen 1

SCF: stem cell factor

ScRNA-seq: single cell RNA sequencing

TCF-1: T cell factor 1

TCR: T cell receptor

TGFβ: transforming growth factor-beta

Th: T helper

TLRs: Toll-like receptors

TNF-α: tumour necrosis factor-alpha

TOX: Thymocyte selection associated high-mobility group box protein

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

TSLP: thymic stromal lymphopoietin

VIP: vasoactive intestinal peptide

β2AR: β2-adrenergic receptor

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Dedication

To my beloved parents.

Chapter 1: Introduction

1.1 The Immune System

1.1.1 The Adaptive Immune System Development

The immune system includes adaptive and innate immune systems. The adaptive immune system consists of B and T cell lymphoid lineages, that mediate antigen specific responses through their antigen specific receptors. Recombination activating gene enzymes (RAG-1 and 2) mediate V(D)J recombination and generation of these antigen specific receptors.

B cells are generated in the bone marrow (BM), express cell surface Ig, CD19, B220 and CD20, and mediate humoral immune response through generation of antibodies. B cells are divided into B-1 and B-2 (conventional) B cells. B-1 B cells are generated during fetal and neonatal period (Montecino-Rodriguez and Dorshkind, 2012). They are self-renewing innate-like B cells, which are mainly found in the peritoneal and pleural cavities. They have more limited antigen-receptor diversity than conventional B cells and constitutively produce natural antibodies (Cerutti et al., 2011). Natural antibodies, present in the circulation, recognize antigens commonly expressed by pathogenic viruses and bacteria such as capsular polysaccharide antigens and play important roles in controlling infections. Conventional B cells include follicular and marginal zone B cells that mainly develop from adult BM (Busslinger, 2004; Allman et al., 1999; Welinder et al., 2011). Conventional B cells are dependent on the transcription factor PU.1, B-Cell-Activating Factor (BAFF) and IL-7 signaling for their development, whereas all these are dispensable for the generation of B-1 B cells.

T cells are generated in the thymus from a BM precursor population that migrates from the BM to the thymus via the blood (Shortman and Wu, 1996; Rothenberg, 2012, 2014). In mice, the

thymus has the highest rates of T cell generation in the first 3-4 weeks of life. After puberty, the thymus gradually shrinks and the generation of new T cells becomes limited. Therefore, the peripheral pool of T cells is maintained by long-lived T cells through adulthood. After settling into the thymus, BM precursors receive Notch signaling that instructs commitment to the T cell lineage (Pui et al., 1999; Radtke et al., 2010, 1999). Notch signaling first induces the expression of the transcription factor T cell factor 1 (TCF-1) (Weber et al., 2011; Germar et al., 2011; Obaldia and Bhandoola, 2015). TCF-1 up-regulates the expression of the transcription factors GATA-binding protein 3 (GATA-3) and B cell leukemia/lymphoma 11B (BCL11b) that are essential for the T-cell-lineage development as well as upregulation of the components of the T cell receptor (TCR) complex. Notch signaling also regulates choices between the αβ vs γδ T cell lineages (Ciofani et al., 2006; Borowski et al., 2002; Kreslavsky et al., 2010; Lauritsen et al., 2006). In addition to the Notch signaling, IL-7 signaling is also essential for T cell development. T cell development is severely blocked in the absence of IL-7, or IL-7 receptor α (CD127) or common cytokine receptor γ chain (γc) or JAK3 (Akashi et al., 1998). After BM precursors' initial differentiation phase into the T lineage pathway, these cells proliferate to develop into double-negative (DN) thymocytes, which do not express CD4, CD8 or CD3:TCR complex, and later give rise to γδ and αβ T cells (Koch and Radtke, 2011; Shah and Zúñiga-Pflücker, 2014; Krueger et al., 2017; Bhandoola et al., 2007). T cells expressing γδ receptors lack the expression of CD4 and CD8 co-receptors, develop during the fetal and neonatal period and mostly home to mucosal tissues (Dunon et al., 1997). They acquire a defined effector function during their development in thymus (Narayan et al., 2012). Considering the difficulties in identifying the ligands that bind γδ receptors, the functions of γδ T cells has remained unclear. DN thymocytes that commit to the αβ lineage choice, gradually develop into CD4 and CD8 expressing double-positive (DP) thymocytes, later mature into CD8 or

CD4 single-positive (SP) thymocytes, and leave the thymus to form functionally distinct subsets in the periphery, namely CD8 and CD4 T cells (Starr et al., 2003; Klein et al., 2014). Additional populations that derive from DP thymocytes are regulatory T (Treg), natural killer T (NKT) and mucosal-associated invariant T (MAIT) cells (Godfrey et al., 2015). Treg cells express the transcription factor forkhead box P3 (FOXP3) and depend on IL-2 signaling for their development (Ohkura et al., 2013). NKT cells express NK receptors, some of them also express CD4, but they are mostly negative for CD4 and CD8 expression (Benlagha et al., 2005). NKT cells differ from other αβ T cells, in recognizing CD1-bound glycolipid antigens rather than major histocompatibility complex class (MHC) molecules for their development and function (Brigl et al., 2003; Rossjohn et al., 2012). Early life composition of the gut microbiome is considered an important source of these agonist lipids that regulate the development of NKT cells (An et al., 2014). NKT cells also acquire effector programs during their development in the thymus. MAIT cells require a non-classical MHC molecule named MHC-related protein 1 (MR1) for their development and function (Seach et al., 2013; Garner et al., 2018). These T cells express an invariant TCRα chain that pair with a limited repertoire of β chains. MAIT cells recognize and become activated by bacterial and yeast derived metabolites that are presented by MR1 molecules. The γδ T, NKT and MAIT cell populations are all considered components of both the adaptive and innate immune system.

As mentioned, TCRαβ T cells are divided into functionally distinct CD8 cytotoxic and CD4 helper T cells. TCF-1 expression in DP thymocytes plays critical role in regulating the development of CD8 versus CD4 T cells. TCF-1 positively regulate the transcription factors GATA-3 and ThPOK (*Zbtb7b*), both of which are required for the development CD4 T cells (Steinke et al., 2014; Wang et al., 2008). In addition, TCF-1 collaborates with the runt-related

transcription factors (RUNX) transcription factors family, which are required for the development of CD8 T cells, and repress CD4 T cell associated genes (Woolf et al., 2003). The development of cytotoxic CD8 T cells further requires the transcription factors EOMES and T-BET (Zhang and Bevan, 2011). CD4 T cells, upon activation, differentiate into distinct cytokine secreting T helper (Th) groups, namely Th1, Th2, Th9, Th17 and follicular Th cells (O'Shea and Paul, 2010). CD4 T cell differentiation into Th1 cells, which produce interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), requires the transcription factors T-BET and RUNX. The differentiation of CD4 T cells into Th2 cells, which produce IL-4, IL-5 and IL-13, requires GATA-3. CD4 T cells differentiation into Th17 cells, which produce IL-17 and IL-22, requires RUNX and RORγt. The transcription factors involved in the development of CD4 Th subsets are discussed in more detail in section 1.5 of this chapter.

1.1.2 The Innate Immune System

Innate immune responses are the first line of defense upon infections (Rivera et al., 2016; Baccala et al., 2009; Brubaker et al., 2015; Mogensen, 2009; Thaiss et al., 2016; Akira et al., 2006; Geering et al., 2013; Stone et al., 2010; Mildner and Jung, 2014; Merad et al., 2013). Most of the cells of the innate immune system are from the myeloid lineage that includes granulocytes (collective term for neutrophils, eosinophils, and basophils), macrophages, mast cells and dendritic cells (DCs). Granulocytes and macrophages provide phagocytic functions. Neutrophils are the most numerous innate immune cell population with prominent phagocytic capabilities. Eosinophil and basophil granules contain a variety of toxic proteins and enzymes, required for the defense against parasitic infections, which also contribute to the development of allergic inflammation. Mast cells are also important contributors to the protection against parasitic worms and development of allergic

reactions. Immature DCs migrate from BM to peripheral tissues, where they perform phagocytosis and micropinocytosis as sensor cells. Thereafter, they can migrate into lymphoid tissues to shape adaptive immune responses. Innate immune recognition of the microbial products occurs through the recognition of microbial-associated molecular patterns by pattern recognition receptors including phagocytic receptors, G-protein-coupled receptors, Toll-like receptors (TLRs), NOD-like receptors and RIG-I-like receptors.

In addition to myeloid cells, the innate immune system includes a family of innate lymphocytes that also provide protection early during infections. These cells have lymphoid features but lack the expression of antigen receptors. They are stimulated by cytokines produced by non-immune and immune cells that have sensed microbial infection or tissue damage.

1.2 The Family of Innate Lymphocytes

The family of innate lymphocytes includes cytotoxic natural killer (NK) cells and cytokine producing innate lymphoid cells (ILCs) (Koyasu et al., 2018; Diefenbach et al., 2017; Diefenbach, 2015; Diefenbach et al., 2014; Eberl et al., 2015; Klose and Artis, 2016; Artis and Spits, 2015). Cytokine producing ILCs are divided into three groups based on their developmental and functional similarities to the main groups of Th cells, namely group 1 ILCs (ILC1s), group 2 (ILC2s) and group 3 (ILC3s). Mouse parabiosis studies have indicated that ILCs are tissue resident cells (Gasteiger et al., 2015).

All innate lymphocytes depend on the γc and JAK3 for their development and maintenance (Robinette et al., 2018; Colonna, 2018). These cells can develop in *Rag1*$^{-/-}$ mice. However, studies with RAG-1 lineage tracer mice show that NK, ILC2Ps and ILC2s transiently express *Rag1* during their development (Yang et al., 2011; Karo et al., 2014; Ghaedi et al., 2016). Transient *Rag*

expression during NK cell development endow them with functional fitness, as *Rag* deficient NK cells are hyper-responsive, short-lived and cannot generate long-lived NK memory cells (Karo et al., 2014). Innate lymphocytes specialize for their tissue-specific functions during their development/maturation within tissues. In addition, ILC lineage representation shows clear differences among different tissues.

The chromatin regions accessible to transcription factors that serve as enhancers or repressors of gene expression are called regulomes and are defined by the assay for transposase-accessible chromatin-sequencing. Regulomes that dictate ILC and Th cell lineage specification are different from each other. In addition, ILC regulomes are mainly bound by E26 transformation specific (ETS) and RUNX transcription factors (Koues et al., 2016; Ebihara et al., 2015; Zook et al., 2016), whereas Th cell regulomes are bound by interferon-regulatory factor (IRF) and activator protein 1 (AP1). ILC and Th cells use similar regulatory regions to control type 1, 2, and 3 functional polarizations including the expression of their signature transcription factor and cytokine genes. These regulatory regions are accessible in ILCs, whereas they are inaccessible in naïve T cells until these cells are primed, activated and polarized (Shih et al., 2016).

1.2.1 Cytotoxic NK Cells

NK cells are present in the BM, blood, spleen, lymph nodes (LN), lung and liver. They are the innate counterparts of CD8 T cells and similarly protect against viral infections and malignant cellular transformation by their capacity to produce IFN-γ, TNFα, perforin and granzymes (Vivier et al., 2008). To perform their functions, NK cells express a variety of activating and inhibitory receptors, and integrate the signals received through these receptors. They express natural

cytotoxicity receptor 1 (NKp46), NK1.1 (in C57BL/6 mice), Fc receptors, NKG2 and Ly49 receptors. It is notable that every NK cell only expresses a fraction of these receptors.

NK cell differentiation starts when common lymphoid progenitors (CLPs) develop into pre-NK cell precursors (pre-NKP) defined as $Lin^{-}CD244^{+}CD127^{+}Flt3^{-}$IL-2 receptor β $(CD122)^{-}$ (Carotta et al., 2011; Geiger and Sun, 2016). Pre-NKP first generate NK cell precursors (NKP) defined as $Lin^{-}CD122^{+}$ (Rosmaraki et al., 2001; Fathman et al., 2011). These NKPs develop into immature NK cells, that are $CD127^{-}NK1.1^{+}$ that later gain expression of CD11b, CD49b (DX5) and Ly49 receptors and functional competence to produce IFN-γ and become cytotoxic. The peripheral NK cell pool can be divided into subsets based on the expression of CD27 and CD11b (Kim et al., 2002; Hayakawa and Smyth, 2006; Chiossone et al., 2009), with the immature NK cells defined as $CD27^{-}CD11b^{-}$ and mature NK cells as $CD27^{-}CD11b^{+}$, suggesting that NK cell maturation occurs in the periphery. Through pro-inflammatory cytokine exposure or viral infection, NK cells differentiate into effector and memory NK cells (Min-Oo et al., 2013; Kamimura and Lanier, 2015). During viral infection, CD8 T cells develop into $KLRG1^{+}$ short-lived and $KLRG1^{-}$ effector subsets (Kaech and Wherry, 2007). The $KLRG1^{-}$ CD8 T cells generate memory CD8 T cells. $KLRG1^{-}$ NK cells also seem to have a greater potential to give rise to long-lived memory NK cells. In addition, NK cells with a history of *Rag1* expression are more likely to develop into $KLRG1^{-}$ NK cells and generate memory NK cells (Karo et al., 2014).

NK cells are dependent on the transcription factors nuclear factor interleukin-3 (NFIL3; also known as E4-binding protein 4), PU.1, Inhibitor of DNA-binding 2 (ID2), Thymocyte selection associated high-mobility group box protein (TOX) and EOMES for their development (Geiger and Sun, 2016). PU.1, a member of the ETS family of transcription factors, is required for the development of B, T, myeloid and NK cells. ETS-1 and myeloid ELF1-like factor are other

members of this family that are required for proper NK cell development (Barton et al., 1998; Ramirez et al., 2012; Lacorazza et al., 2002). NFIL3 is critical at the NKP stage for NK cell lineage commitment (Gascoyne et al., 2009; Kamizono et al., 2009; Yang et al., 2015a). NFIL3 deficient mice lack NK cells, whereas they have normal B and T cell development. The transcription factor RUNX3 and RUNX common obligatory partner (CBF-β) are required early during NK cell development and also for mature NK cells maintenance (Ohno et al., 2008; Guo et al., 2008). ID2 (Yokota et al., 1999; Boos et al., 2007; Schotte et al., 2010) and TOX (Aliahmad et al., 2010) are required for development of immature NK cells into mature cells. EOMES facilitate the acquisition of DX5 and Ly49 receptors (Townsend et al., 2004; Gordon et al., 2012).

IL-15 is required for NK cell development (Geiger and Sun, 2016; Ranson et al., 2003; Vosshenrich et al., 2005; Cooper et al., 2002; Marçais et al., 2014; Orange et al., 1996), while IL-7 is not. IL-12 is required for NK cell IFN-γ production, expansion and memory generation. IL-18, type I IFNs and IL-33 are important for optimal expansion of these cells.

1.2.2 Cytokine Producing ILCs

1.2.2.1 ILC1s

ILC1s are considered to be the innate equivalents of Th1 cells and are similar to the previously described immature TRAIL^{+}CD49a^{+}CD49b(DX5)$^{-}$ NK cells (Takeda et al., 2001, 2005; Daussy et al., 2014). ILC1s mostly develop during the fetal and neonatal period (Constantinides et al., 2015) and are found in the liver, gut (Klose et al., 2014; Constantinides et al., 2014), salivary glands (Cortez et al., 2016) and adipose tissue (Sullivan et al., 2016). An ILC1 precursor is also characterized in the BM (Klose et al., 2014). ILC1s express T-BET, but not EOMES, and are dependent on T-BET and RUNX for their development (Gordon et al., 2012; Fuchs et al., 2013;

Klose et al., 2014; Daussy et al., 2014). ILC1s produce IFN-γ, TNFα and TRAIL, but not perforin and granzymes (Cortez and Colonna, 2016; Colonna, 2018). ILC1s share close developmental, phenotypic and functional similarities with NK cells. They express NK1.1 and NKp46 and are dependent on IL-15, but not IL-7, for their development (Klose et al., 2014; Gronke et al., 2016). ILC1s are important in the efficient control of intracellular parasites including *Toxoplasma gondii*. ILC1 tissue residency program is driven by the transcription factor HOBIT and TGFβ signaling (Mackay et al., 2016). It is notable that HOBIT is also expressed by human NK cells and required for their development (Post et al., 2017). IL-15, IL-12 and IL-18 promote immediate ILC1 activation during infection (Weizman et al., 2017; Klose et al., 2014). The inhibitory molecules expressed by ILC1s include the ITIM receptor CD200R (binds CD200), TIGIT and CD96 (both bind CD155) (Cortez and Colonna, 2016; Colonna, 2018).

1.2.2.2 ILC2s

ILC2s are the innate equivalents of Th2 cells (Koyasu et al., 2018). The phenotypic and functional characterization of ILC2 as well as their heterogeneity and developmental requirements are discussed in detail below.

1.2.2.3 ILC3s

ILC3s are the innate equivalents of Th17 cells. They express the transcription factor RORγt, on which they are dependent for their development. They are stimulated by IL-23, IL-1α and IL-1β and produce IL-17A/F and IL-22 (Pickert et al., 2009; Reynders et al., 2011; Hernández et al., 2015; Gronke and Diefenbach, 2015; Gronke et al., 2016; Branzk et al., 2018; Lindemans et al., 2015). IL-17 induces neutrophilia and IL-22 promotes epithelial cell production of Reg3

antimicrobial peptides, protection against viral and bacterial infections, tissue regeneration and wound healing.

ILC3s are heterogeneous. ILC3s that emerge during the fetal period and mediate the development of LNs and Peyer's patches are called lymphoid tissue inducer (LTi) cells (Mebius et al., 1997; Yoshida et al., 1999; Eberl et al., 2004; Eberl, 2005). LTi cells express lymphotoxin (LT) α1β2 and activate LTβR expressing stromal cells, which leads to recruitment of innate and adaptive immune cells and formation of lymphoid organs. In adult mice, at least two distinct ILC3 subsets are present based on the expression of CCR6 and T-bet. CCR6^{+}T-bet^{-} ILC3s resemble LTi cells and produce IL-17A/F and IL-22 upon activation. These cells express G-protein-coupled receptor 183 and migrate toward 7α,25-hydroxycholesterol produced by stromal cells in isolated lymphoid follicles (ILFs) (Emgård et al., 2018). CCR6^{+} ILC3s express receptor activator of nuclear factor κB ligand also known as TNF superfamily member 11 (RANKL or TNFSF11) and its receptor RANK (TNFRSF11a) (Bando et al., 2018). RANK-RANKL interactions between ILC3s inhibit their activation and proliferation to regulate their function and population size. CCR6^{-}T-bet^{+} develop during the first weeks after birth, are mostly found in the intestinal lamina propria, and can be divided into two subsets based on the expression of NKp46 (Gronke et al., 2016; Branzk et al., 2018; Klose et al., 2013, 2014; Rankin et al., 2013; Sanos et al., 2009; Luci et al., 2009; Satoh-Takayama et al., 2008; Vonarbourg et al., 2009). CCR6^{-}T-bet^{+} ILC3s that express NKp46 and NKG2D, produce IL-22 and IFNγ but not IL-17A/F. T-bet and Notch signaling can instruct downregulation of the RORγt expression and their differentiation into IFNγ and TNFα producing ILC1s (also known as ex-ILC3s) (Klose et al., 2013; Rankin et al., 2013; Viant et al., 2016; Chea et al., 2016a; Klose et al., 2014). It is notable that these ex-ILC3s can reverse this plasticity by re-gaining RORγt expression and losing NKp46 (Bernink et al., 2015; Rankin et al., 2016).

Nutrient signaling plays important roles in the development of fetal and neonatal lymphoid organs and immune fitness during adulthood. Vitamin A metabolite retinoic acid (RA) directly regulates RORγt expression and the population size of fetal LTi cells, and hence the development of LNs and Peyer's patches (Van De Pavert et al., 2014). Vitamin A deficiency results in fewer and smaller LNs and PPs and reduced immune fitness later in life. RA also control adult ILC3 population size and their production of IL-22 (Spencer et al., 2014). RA deficiency results in compromised immunity to the attaching effacing bacterial pathogen *Citrobacter rodentium.* However, RA deficiency results in increased ILC2 number and activity and therefore immunity to helminth infections. Aryl hydrocarbon receptor (AhR), is a transcription factor that is also activated by dietary (including flavonoids and glucosinolates found in plants), bacterial and endogenous ligands. Signals from AhR are required for the postnatal maintenance and expansion of $CCR6^{-}$ ILC3s, formation of the ILFs, proper IL-22 production and defense against infection with *C. rodentium* (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2011; Kiss and Diefenbach, 2012). In addition, AhR signaling inhibit ILC2 activation and hence regulates ILC2 and ILC3 balance in the intestine (Li et al., 2018).

Peripheral nervous system can affect ILC functions. ILFs are innervated by the peripheral enteric nerves that are sheathed in glial cells. These glial cells sense microbial presence through TLR signaling and release glial cell-derived neurotrophic factor family of ligands (GFLs). ILC3s express the receptor tyrosine kinase RET that binds GFLs (Ibiza et al., 2016), resulting in their proper production of IL-22. In addition, after *Escherichia coli* infection, the vagal system release of acetylcholine stimulates ILC3s resulting in their production of the protectin conjugates in tissue regeneration 1,which mediates resolution of inflammation and tissue repair (Dalli et al., 2017).

ILC3s also play critical roles in the immune responses mediated by adaptive B and T cells. ILC3s are critical for the effective production of immunoglobulins by B cells, through their production of a variety of TNF superfamily ligands including LTα1β2, LTα3, BAFF, RANKL, LIGHT, APRIL and CD40L (Tsuji et al., 2008; Kruglov et al.; Magri et al., 2014; Magri and Cerutti, 2015). ILC3s express MHC II and present commensal bacteria-specific antigens to T cells, which inhibit T cell activation and promote immune tolerance (Hepworth et al., 2013, 2015). ILC3-derived granulocyte-macrophage colony-stimulating factor controls the colonic DCs and macrophages maintenance and functions that is required for inducing Treg cells and oral tolerance (Mortha et al., 2014).

1.3 ILC2s

1.3.1 ILC2 Phenotype and Function

ILC2s mostly develop during neonatal period and can be found in the lung, gut, skin and adipose tissue (Bando et al., 2013; de Kleer et al., 2016; Saluzzo et al., 2017; Steer et al., 2017; Ghaedi et al., 2016; Hoyler et al., 2012). The main key cytokines that induce activation and effector functions of ILC2s include thymic stromal lymphopoietin (TSLP), IL-2, IL-7, IL-25 and IL-33 (Van Dyken et al., 2016). ILC2s can be identified by a combination of cell surface markers, although their surface phenotypes vary depending on their tissue origin. Tissue-derived signals that are independent of the microbiota and cytokine signaling induce their tissue-specific gene expression profiles (Ricardo-Gonzalez et al., 2018; Zhu, 2018). IL-33R (ST2) is preferentially expressed on lung (Ricardo-Gonzalez et al., 2018; Halim et al., 2012b; a) and adipose tissue ILC2s (Molofsky et al., 2015), whereas IL-25R is expressed by gut ILC2s (von Moltke et al., 2015; Howitt et al., 2016; Gronke and Diefenbach, 2016). Moreover, skin ILC2s express IL-18Rα, and IL-18 is

required for their optimal activation, proliferation and function. It is notable that adult BM contains committed ILC2 precursors, termed ILC2 progenitors (ILC2Ps) (Halim et al., 2012b; Hoyler et al., 2012) and defined as $Lin^{-}GATA\text{-}3^{+}Thy1^{+}CD127^{+}ST2^{+}CD25^{+}KLRG1^{-}$. They produce low amounts of IL-5 and IL-13 upon stimulation with phorbol myristate acetate (PMA) and ionomycin compared to lung ILC2s. In addition, upon transplantation into alymphoid mice, they efficiently differentiate into ILC2s in the mucosal tissues.

In mouse lungs, ILC2s can be defined as lineage marker negative $(Lin)^{-}$, positive for Thy1 (CD90), IL-7 receptor α (IL-7Rα) (CD127), ST2 and IL-2Rα (CD25). These cytokine receptors are important stimulatory receptors for ILC2s (Martinez-Gonzalez et al., 2015; Van Dyken et al., 2016; Martinez-Gonzalez et al., 2016). ILC2s express the transcription factors GATA3 and RORα, which are critically required for ILC2 development (Halim et al., 2012b; Wong et al., 2012; Hoyler et al., 2012; Mjösberg et al., 2012; Yagi et al., 2014), and are negative for the expression of the transcription factors Eomes, T-bet and RORγt.

Lung ILC2s are stimulated in vivo by inhaled allergens or IL-33 and they produce large amounts of IL-5 and IL-13 (Halim et al., 2012a; Chang et al., 2011), which induce T cell-independent eosinophilia, goblet cell hyperplasia, mucus hyperproduction, and airway hyperreactivity. ILC2s express the enzyme arginase-1 (Arg1), as a key regulator of their metabolic programming (Monticelli et al., 2016). Expression of Arg1 by ILC2s is required for proper arginine catabolism, polyamine biosynthesis and aerobic glycolysis necessary for their proliferation and pro-inflammatory function after activation. Allergen-experienced lung ILC2s acquire antigen non-specific memory properties. They upregulate IL-25R, persist, and mediate an enhanced type 2 inflammation upon re-activation by unrelated allergens (Martinez-Gonzalez et al., 2018, 2016). ILC2s also promote allergen-induced Th2 cell differentiation and memory response (Halim

et al., 2014; Martinez-Gonzalez et al., 2015; Halim et al., 2016), thus have important roles in both T cell independent and dependent allergic lung inflammation. Activated ILC2s also produce the growth factor amphiregulin, a ligand of the EGF receptor (Monticelli et al., 2011, 2015) to promote airway and intestinal epithelial growth and therefore tissue repair after influenza virus infection or intestinal damage. In addition ILC2s produce IL-4 (Pelly et al., 2016), IL-2 (Pelly et al., 2016), IL-9 (Wilhelm et al., 2011) and IL-10 (Seehus et al., 2017) as well as methionine enkephalin peptides (Brestoff et al., 2015; Lee et al., 2015; Flach and Diefenbach, 2015). They express cysteinyl leukotriene receptor 1 (CysLT1R) (Doherty et al., 2013), DR3 (TNFRSF25) (Meylan et al., 2014), GITR (TNFRSF18)/Ligand (TNFSF18) (Nagashima et al., 2018; Rauber et al., 2017), ICOS/Ligand (Maazi et al., 2015; Molofsky et al., 2015; Rauber et al., 2017) and OX40 ligand (Halim et al., 2018). ILC2s express a variety of inhibitory receptors including the ITIM-bearing receptors KLRG1 that binds to E-cadherin (Taylor et al., 2017a; Vivier, 2017) and programmed death 1 (PD-1) that binds to PD-1 ligand (PD-L1) (Taylor et al., 2017b; Yu et al., 2016). They express the receptors for and are inhibited by the cytokines IL-27, IFNα, β and γ (Moro et al., 2016; Molofsky et al., 2015; Duerr et al., 2015). ILC2s also express the receptor and are inhibited by prostaglandin I_2 (Zhou et al., 2016).

ILC2s are in close proximity to peripheral nerves that can affect their functions. Vasoactive intestinal peptide (VIP) is a neuropeptide that binds VIP receptor on ILC2s and enhance their cytokine production during helminth infections (Nussbaum et al., 2013). Neuromedin U is another neuropeptide that can bind the neuromedin receptor 1 on ILC2s resulting in their activation (Klose et al., 2017; Wallrapp et al., 2017; Cardoso et al., 2017). In addition, lung ILC2s reside in close proximity to pulmonary neuroendocrine cells that can release the neuropeptide calcitonin gene-related peptide to stimulate ILC2s and amplify allergic responses (Sui et al., 2018). In contrast to

these activating receptors, ILC2s express the β2-adrenergic receptor (β2AR) (Moriyama et al., 2018). β2AR deficiency results in exaggerated ILC2 responses. β2AR agonist treatment inhibits ILC2s activation and proliferation. Hence β2AR pathway presents a neuronal-derived negative regulator of ILC2s.

Human ILC2 are defined as $Lin^{-}CD127^{+}$ that are positive for chemoattractant receptor-homologous molecule expressed on TH2 cells $(CRTH2)^{+}$ (Mjösberg et al., 2011, 2012; Monticelli et al., 2016; Xue et al., 2014; von Moltke et al., 2017). They also express GATA-3, IL-25R, IL-33R, CD161 and Arg1, and produce IL-5 and IL-13 upon stimulation. The expression of CRTH2 by ILC2s reflect the ability of these cells to sense lipid mediators including prostaglandin D2 released during inflammation. In addition, human ILC2s express the receptors PTGER2 and PTGER4 and are inhibited by prostaglandin E2 (Maric et al., 2018). All these receptors/ligands on ILC2s enable them to interact with a variety of cell types and participate in a broad range of immune responses. However, it is unknown whether a single population of ILC2s produces all these molecules.

1.3.2 ILC2 Heterogeneity

Until recently, the ILC2 population was thought to be homogeneous. Although it has been reported that intraperitoneal injection of IL-25 or helminth infection induces a population of $KLRG1^{hi}IL\text{-}25R^{+}$ ILC2s, termed inflammatory ILC2s, in the lung (Huang et al., 2018b, 2015), these cells are not lung resident at resting state. Instead they migrate from intestinal lamina propria to peripheral sites including the lung. Single cell RNA sequencing (scRNA-seq) analysis of human $Lin^{-}CD45^{+}CD127^{+}$ ILCs in tonsil revealed distinct sub-populations of ILC3s (Björklund et al., 2016), whereas no heterogeneity among ILC2s was found. Moreover, scRNA-seq analysis of

$Lin^{-}CD127^{+}$ ILCs in the small intestine of mice divided the ILC1, ILC2 and ILC3 populations into distinct transcriptional states (Gury-BenAri et al., 2016). Intestinal ILC2s had four transcriptional states designated ILC2a-d, based on graded expression of *Gata3* and *Klrg1*. Flow cytometric analysis showed that >90% of intestinal ILC2s are $KLRG1^{+}$ and included $IL\text{-}5^{+}IL\text{-}4^{-}$ cells, whereas the small $KLRG1^{-}$ subset included rare $IL\text{-}5^{-}IL\text{-}4^{+}$ cells. In a more recent study, scRNA-seq analysis was performed on $Il5^{+}$ ILC2s from the lung, gut, fat and skin as well as $Arg1^{+}$ cells from BM (Ricardo-Gonzalez et al., 2018; Zhu, 2018). All skin and ~10% lung $Il5^{+}$ ILC2s as well as less than 5% BM $Arg1^{+}$ cells expressed IL-18Rα, but not ST2. IL-18 was required for the optimal IL-5 and IL-13 production and proliferation of skin ILC2s in a model of atopic dermatitis. In contrast, lung $Il5^{+}$ ILC2s upon *in vitro* stimulation with IL-18 produced barely detectable amounts of IL-13. In addition, intradermal injection of IL-18 resulted in minimum *Il13* expression by lung $Il5^{+}$ ILC2s. The biological significance and the developmental pathways of the $IL\text{-}18R\alpha^{+}ST2^{-}$ ILC2 subset in the lung and BM are unknown. Another recent analysis of human ILC2s in various tissues by mass cytometry has shown extensive heterogeneity among individuals and tissue origins (Simoni et al., 2017). Human peripheral blood ILC2s were $IL\text{-}18R\alpha^{+}$ and they produced IL-5 and IL-13 upon stimulation with IL-18. However, it is unknown whether IL-18Rα is expressed by a subset of human ILC2s or on previously activated ILC2s. Although, these studies have shown that the ILC2 population may be more complex than previously thought, the functional significance and physiological relevance of ILC2 heterogeneity remains largely unknown.

1.3.3 ILC2 Plasticity

Human ILC2s can trans-differentiate into IFNγ producing ILC1s under the influence of IL-1β or IL-33 and IL-12 signaling. IL-1β induces ILC2 proliferation and cytokine production. IL-1β also

induces the transcriptional activation of the NK/ILC1-associated loci encoding T-bet, IL-12Rβ1 and IL-12Rβ2 in ILC2s, which enables them to respond to IL- 12 and convert into IFNγ producing ILC1s (Ohne et al., 2016; Bal et al., 2016; Lim et al., 2016). Most of these IFNγ expressing ex-ILC2s also express IL-13. The importance of IL-12 signaling in ILC2s/ILC1 conversion is confirmed by the analysis of individuals with Mendelian susceptibility to mycobacterial disease due to lack of IL-12Rβ1. In these individuals, ILC2s fail to trans-differentiate into ILC1s. The increase in the T-bet expression by trans-differentiated ILC2s is correlated with downregulation in the expression of GATA-3, CRTH2, IL-5 and IL-13. These ex-ILC2s accumulate in lungs of individuals with the inflammatory disease severe chronic obstructive pulmonary disease (COPD). In addition, IL-18 and IL-12 can promote conversion of mouse ILC2s into ILC1, and this conversion is boosted in the presence of IL-33 (Silver et al., 2016). This trans-differentiation of lung ILC2s occurs in mice after influenza virus infection. Both IL- 33 and IL- 1β belong to the IL-1 family of cytokines and can activate NF-κB signaling that might be crucial for ILC2 trans-differentiation to ILC1s.

1.4 Development of Innate Lymphocytes

1.4.1 Classical Model of Lymphopoiesis

Haematopoietic stem cells (HSC) are a fraction of $Lin^{-}EPCR^{+}CD48^{-}CD150^{+}$ cells in the haematopoietic organs including BM and can generate all types of blood cells (Benz et al., 2012). They have self-renewal capacity and differentiate into blood cell lineages by a stepwise loss of their lineage potential through differentiation into consecutive array of progenitors. HSCs initially differentiate into common myeloid progenitors (CMP) and lymphoid primed multipotent progenitors (LMPPs) (Challen et al., 2009; Kondo, 2010). CMPs give rise to

granulocyte/macrophage and megakaryocyte/erythrocyte progenitors and eventually neutrophils, basophils, eosinophils, mast cells, monocytes/macrophages, DCs as well as megakaryocyte/platelets and erythroblasts/erythrocytes. On the other hand, LMPPs represent a progenitor population that is mainly primed for lymphoid lineage development but retain residual myeloid potential (Adolfsson et al., 2005).

In the classical scheme of lymphopoiesis, LMPPs that are identified as $Lin^{-}Kit^{hi}Sca\text{-}1^{hi}Flt3^{+}CD127^{-}$ BM cells first differentiate into CLPs (Kondo et al., 1997; Karsunky et al., 2008) that are identified as $Lin^{-}Kit^{lo}Sca\text{-}1^{lo}Flt3^{+}CD127^{hi}$ cells. CLPs lack myeloid potential and are considered to first develop into committed T, B and ILC progenitors to eventually give rise to T, B and innate lymphocytes. However, the studies on the most immature T cell progenitors in the thymus, termed early thymic progenitors (ETPs), suggested that T cell development does not follow the classical scheme of lymphocyte development (Allman et al., 2003; Bhandoola and Sambandam, 2006). A subset of LMPPs defined by their expression of *Rag1* were more efficient T cell progenitors compared to CLPs in transplantation assays (Igarashi et al., 2002; Perry et al., 2006). Also, LMPPs can circulate in the peripheral blood, whereas CLPs are hardly detectable in the peripheral blood (Schwarz and Bhandoola, 2004). In addition, ETPs have residual myeloid potential. All these results suggest that ETPs and hence T cells develop from LMPPs via a CLP-independent pathway. Later studies showed that CLPs can be divided into two subsets based on the expression of Ly6D (Inlay et al., 2009). $Ly6D^{-}$ CLPs which have residual myeloid and potent T and NK cell potential, were termed all-lymphoid progenitors (ALPs). $Ly6D^{+}$ CLPs that mainly include B cell progenitors, were termed B-cell-biased lymphoid pro-genitors (BLPs).

1.4.2 Common ILC Precursors

All innate lymphocytes including cytotoxic NK cells and helper-like ILCs develop from a common *Tcf7* (TCF-1)-expressing precursor population termed early ILC progenitors (EILPs) (Figure 1.1) (Yang et al., 2015b; Harly et al., 2017; Harly and Bhandoola, 2017). EILPs are identified by Lin^{-}*Tcf7*$^{+}\alpha4\beta7^{+}CD127^{-}Thy1^{-}Flt3^{lo}CD25^{-}CD122^{-}CXCR6^{-}$. In addition to *Tcf7*, EILPs highly express *Nfil3*, *Tox* and *Runx*, which are required for the development of all innate lymphocytes. EILPs are considered specified but not committed progenitors to innate lymphocytes, since they have residual DC potential. It is notable that another common progenitor population to both NK and helper ILC lineages has been identified as $Lin^{-}CD127^{+}CXCR6^{-/+}\alpha4\beta7^{+}Flt3^{-}$ BM cells and termed αLP (Yu et al., 2014; Possot et al., 2011). The $CXCR6^{-}$ αLP have lost the B cell potential but retain T cell and ILC lineage potential. However, the $CXCR6^{+}$ αLP only differentiates into the NK and ILC lineages. It is thought that these common progenitors first differentiate into Pre-NKP/NKP (Geiger and Sun, 2016) and common progenitors for the cytokine-producing (helper-like) ILCs termed CHILPs (Klose et al., 2014; Gronke et al., 2016). As mentioned earlier Pre-NKP/NKP give rise to mature NK cells. CHILPs, are *Id2*-expressing progenitors in the fetal liver and adult BM that give rise to ILC1, ILC2, ILC3 (including LTi cells), but not NK, T or B cells upon transfer into alymphoid mice and in *in* vitro differentiation assays. CHILPs are identified as Lin^{-}*Id2*$^{+}\alpha4\beta7^{+}Thy1^{+}CD127^{+}Flt3^{-}CD25^{-}ST2^{-}$ cells. A fraction of them expresses the transcription factor promyelocytic leukemia zinc finger (PLZF; encoded by the *Zbtb16*). PLZF is expressed in NKT cells and is required for their development (Kovalovsky et al., 2008; Savage et al., 2008). ID2 controls PLZF transcription during NKT cell development (Verykokakis et al., 2013), hence the $PLZF^{-}$ CHILPs might give rise to the $PLZF^{+}$ CHILPs. $PLZF^{+}$ CHILPs were termed common precursors to ILCs (ILCPs) (Constantinides et al., 2014). ILCPs express the transcription factor

genes *Tcf7, Tox, Rora* and *Gata3*. ILCPs had potential to give rise to ILC1, ILC2, ILC3, but not LTi, NK, T or B cells upon transfer into alymphoid mice and in *in* vitro differentiation assays, Hence, ILCPs had more restricted developmental options than CHILPs. In agreement with this, *Zbtb16* lineage tracing marks ILCs, but not NK or LTi cells. It is notable that ILCPs specifically express the cell surface marker PD-1 that allows their isolation without relying on the use of reporter mice (Xu et al., 2015; Yu et al., 2016). All these precursors are present in the fetal liver (Bando et al., 2015; Constantinides et al., 2014, 2015; Klose et al., 2014; Ishizuka et al., 2016; Possot et al., 2011; Cherrier et al., 2012; Chea et al., 2016b; Yoshida et al., 2001). However, ILCs generated during fetal and adult periods are different. During the fetal period, mainly ILC3s and ILC1s develop. Whereas, ILC2s and NK cells are mainly generated after birth. It is notable that the contribution of the aforementioned adult BM precursors to ILC generation is still not clear, considering that ILCs are long lived and seem to be replenished by self-renewal (Gasteiger et al., 2015). In addition, these cells might be replenished by a pool of tissue resident ILC progenitors (Ebihara et al., 2015).

In humans, several ILC-restricted progenitors have been identified (Renoux et al., 2015; Montaldo et al., 2012; Chen et al., 2018; Scoville et al., 2016). A human systemic ILC precursor population in circulation and tissues have also been found, which can give rise to NK cells, ILC1s, ILC2s and IL-22-expressing (but not IL-17A-expressing) ILC3s (Lim et al., 2017b, 2018) *in vivo* in humanized mice and *in vitro* differentiation assays. These systemic ILC precursors were defined as $Lin^{-}CD7^{+}CD127^{+}CD117(Kit)^{+}$. These cells lacked the expression of surface molecules associated with NK cells, ILC1s, ILC2s and ILC3s. They expressed intermediate levels of GATA-3 compared to NK cells and $CRTH2^{+}$ ILC2s and lacked RORγt, T-bet and Eomes. A fraction of these cells expressed PLZF. The transcriptomic and epigenetic analyses of these cells revealed

high expression of *Cd2*, *Cd7*, *Tox*, *Tcf7*, *Runx3*, *Gata3*, *Id2*, *Zbtb16*, *Rorα* as well as *Il18r1* and *Il1r1*. In addition, these cells do not express the key mature ILC-associated-genes including *Eomes*, *Tbx21*, *Rorc*, *Il1rl1*, *Il23r*, *Ifng*, *Il13*, *Il5* and *Il22* but have these genes in an epigenetically poised configuration. These systemic progenitors in circulation and in tissues may differentiate into ILCs within tissues in response to the local tissue microenvironment signals generated during homeostatic and inflammatory conditions.

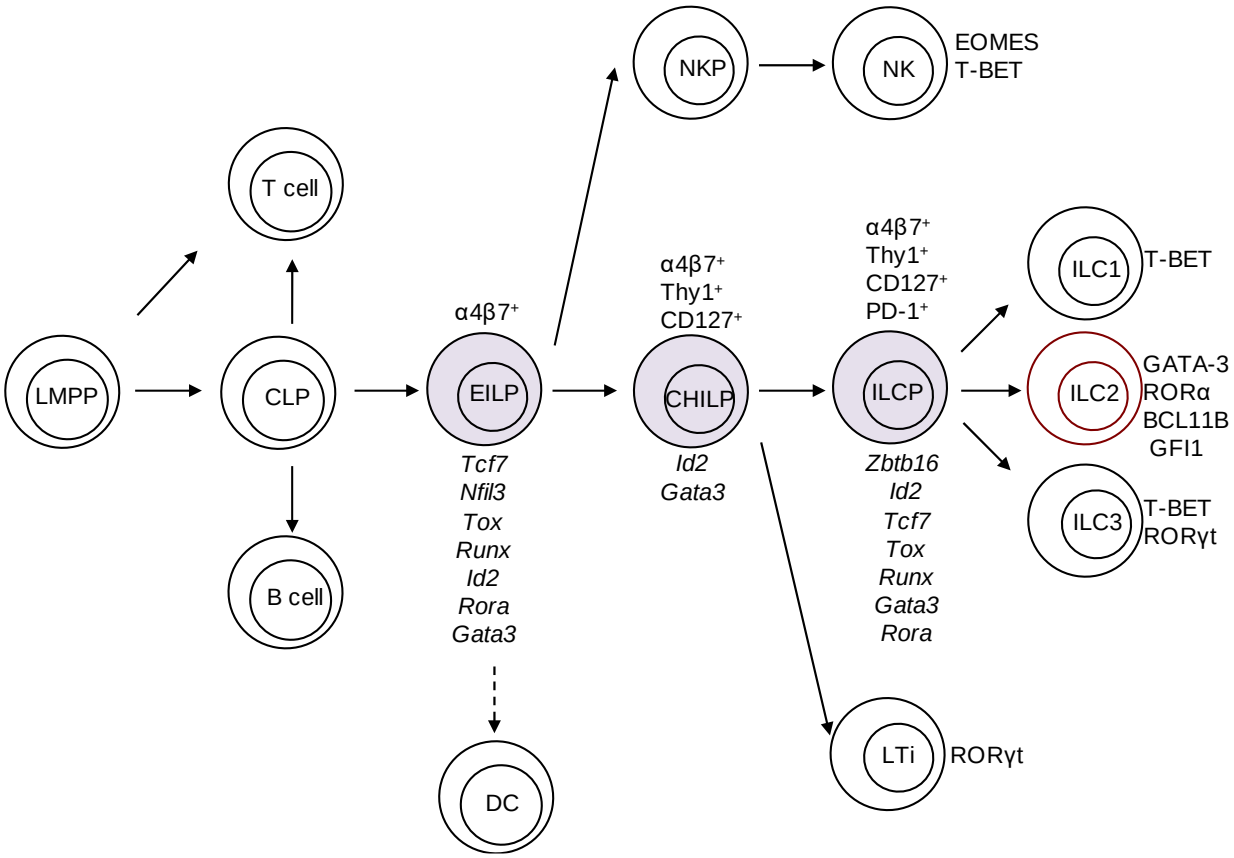


Figure 1.1 LMPPs Give Rise to CLPs, Which Are Perceived to Give Rise to All Lymphocytes
T cell development does not require a CLP stage of development. In addition, it is generally perceived that EILPs develop from CLPs. EILPs are specified, but not committed progenitors to innate lymphocytes since they can differentiate into DCs. EILPs differentiate into NKP and CHILPs. NKPs give rise to cytotoxic NK cells. CHILPs give rise to both LTi cells and ILCPs. ILCPs are the common precursors to ILC1s, ILC2s and ILC3s. On the top of each developmental stage the cell surface proteins expressed are listed. On the bottom, the transcription factors expressed are listed. The main transcription factors expressed by ILC1s, ILC2s and ILC3s are also listed.

1.5 Transcription Factors Regulating ILC Lineage and ILC2 Differentiation

1.5.1 NFIL3

NFIL3 is a basic region leucine zipper transcription factor and a key regulator of both the NK cell and helper ILC lineages (Yu et al., 2014; Xu et al., 2015; Seillet et al., 2014, 2016; Geiger et al., 2014; Kamizono et al., 2009; Male et al., 2014). EILPs express *Nfil3* as well as low levels of *Id2* (Yang et al., 2015b). NFIL3 binds and regulates Id2 locus. NFIL3 is required for the development of αLPs, NKPs, CHILPs, ILCPs and ILC2Ps. Consequently, NFIL3 deficiency results in lowered frequencies and absolute numbers of NK cells, ILC1s, ILC2s and ILC3s. IL-7 signaling is speculated to be involved in the regulation of NFIL3. It is notable that only a transient expression of NFIL3 prior to ID2 is required for the innate lymphoid lineage specification and development. Deletion of NFIL3 after the induction of ID2 expression has no effect on the development and maintenance of these cells. NFIL3 was also shown to bind the TOX promoter, another transcription factor involved in early innate lymphoid lineage specification and development, in a mouse lymphoma cell line. During NK cell development NFIL3 also directly binds the regulatory region of EOMES and promote its transcription. In addition, NFIL3 is required for the proper development of Peyer's patches, but not LNs. NFIL3 deficiency results in reduced number of Peyer's patches as well as impaired recruitment and distribution of lymphocytes within them.

1.5.2 ID2

ID2 is strictly required for the development of all innate lymphocytes including cytotoxic NK cells and ILCs (Diefenbach et al., 2014; Kee, 2009; Yokota et al., 1999). ID2 is a Helix-Loop-Helix (HLH) transcriptional repressor, which can heterodimerize with E proteins to repress their transcriptional activity. E proteins are transcription factors containing an HLH domain as well as

a basic DNA-binding region. Mammalian E proteins include E12 and E47, which arise through alternative splicing of the *Tcf3*, HEB (*Tcf12*), E2-2 (*Tcf4*) (Wang and Baker, 2015). E proteins are essential in the T and B cell development. ID2 heterodimerization with E proteins inhibit their DNA binding and transcriptional activity and suppress T and B cell fates. Consistent with the ID2 roles, several genes are upregulated in ID2 deficient αLPs compared to their wild type counterparts that are known targets of E proteins (Seillet et al., 2016). In addition, these cells have lower expression of *Tox*, *Gata3*, *Arg1* and *Lmo4.* Consequently, ID2 expression is not only required for commitment to the innate lymphoid lineage, but also for inducing/maintaining the critical regulators *Tox* and *Gata3*. ID2 is required at different development stages during NK cell and ILC lineage development. NKPs are $ID2^{-}$ and develop in ID2 deficient mice, whereas CHILP and ILCPs express ID2 and are dependent on it for their development (Boos et al., 2007; Yang et al., 2015b; Harly et al., 2017). ID2 is expressed after NK cell lineage specification and is required for their terminal differentiation (Boos et al., 2007; Fathman et al., 2011; Geiger and Sun, 2016). ID2 is also required for LN organogenesis. Notch and TOX signaling might be important in the induction of ID2 in progenitors to innate lymphocytes (Seehus et al., 2015a; Cherrier et al., 2012; Delconte et al., 2016).

1.5.3 TOX

TOX, encoded by the *Tox* gene, is a high mobility group (HMG)-box transcription factor that is required for the development of the $CD4^{+}$ T, Treg and NKT cells (Wilkinson et al., 2002; Aliahmad and Kaye, 2008; Aliahmad et al., 2011). TOX is also required for the development of LTi cells and NK cells (Aliahmad et al., 2010). Hence, $Tox^{-/-}$ mice lack LN organogenesis and mature NK cells, whereas NKPs are present in these mice. EILPs, αLPs, CHILPs and ILCPs express TOX

(Harly et al., 2017; Constantinides et al., 2014; Seehus et al., 2015a; Seillet et al., 2016; Yu et al., 2014). In addition, EILPs and CHILPs fail to develop and ILC1, ILC2 and ILC3 are severely reduced in numbers in *Tox*$^{-/-}$ mice. It is suggested that *Tox* expression in ILC progenitors might be regulated by Notch or NFIL3 (Yu et al., 2014; Seehus et al., 2015b).

1.5.4 TCF-1

TCF-1, encoded by the *Tcf7* gene, is a HMG-box transcription factor. TCF-1 is critical in T cell lineage development and TCF-1 deficiency results in substantially reduced thymic cellularity and peripheral mature T cells (Ohteki et al., 1996; Verbeek et al., 1995; Weber et al., 2011; Germar et al., 2011; Obaldia and Bhandoola, 2015). TCF-1 is directly up-regulated by Notch signaling in ETPs and continues to be expressed during T cell development in thymus as well as mature T cells. TCF-1 induces the expression of the T-cell-lineage essential transcription factors GATA-3 and BCL11b and might also play a role in blocking B cell fate during T cell development. TCF-1 cooperates with lymphoid enhancer binding factor 1 to promote DP thymocytes to a CD4 T cell fate (Steinke et al., 2014). Furthermore, TCF-1 initiates Th2 differentiation by promoting GATA-3 expression and blocking Th1 cell fate (Yu et al., 2009).

During NK cell and ILC development, *Tcf7* expression by EILPs (Yang et al., 2015b; Harly et al., 2017) is consistent with a transient Notch signaling. IL-7 signaling along with a transient Notch signaling in CLPs leads to the generation of ILC2s, whereas sustained Notch signaling in CLPs leads to the generation of T cells (Koga et al., 2018). NFIL3, TOX and TCF-1 are all expressed early during ILC lineage development (Yang et al., 2015b; Seillet et al., 2016; Yu et al., 2014; Seehus et al., 2015a; Harly et al., 2017; Ishizuka et al., 2016). NFIL3 and TOX might be involved in upregulating *Tcf7* expression, and perhaps all the three transcription factors are

required for the efficient generation of EILPs. Therefore, NFIL3-TOX-TCF-1 cascade may provide the early transcriptional machinery required for NK and ILC lineage specification and commitment. *Tcf7*$^{-/-}$ mice have severely reduced αLPs, CHILPs and NKPs (Seillet et al., 2016; Yang et al., 2015b). The remaining *Tcf7*$^{-/-}$ αLPs have upregulated HSC and B cell developmental programs genes including *Hhex*, *Lmo2*, *Spib*, *Irf8* and *Ly6d* (Seillet et al., 2016), suggesting that TCF-1, in addition to ID2, is involved in blocking alternative fate progression. In accordance with T cell development, TCF-1 might activate the expression of GATA-3 and BCL11b during ILC-lineage development (Yang et al., 2013; Seillet et al., 2016). TCF-1 promotes ILC2 development through GATA-3-dependent and -independent pathways. TCF-1 up-regulates *Il17rb* and *Il2ra* expression by ILC2s through a GATA-3-dependent pathway, and directly up-regulates the expression of *Il7r*. *Tcf7*$^{-/-}$ αLPs have lower levels of *Bcl11b* that is specifically required for ILC2 development. Hence, TCF-1 deficiency results in loss of ILC2Ps, ILC2s and compromised immune responses to the nematode *Nippostrongylus brasiliensis* (Mielke et al., 2017; Yang et al., 2013). TCF-1 is also required for the development NKp46^{+}RORγt^{+} ILC3s and hence defense against *C. rodentium* infections (Mielke et al., 2017) as well as proper establishment of Ly49 receptor repertoire on NK cells (Held et al., 2003, 1999). ID2 can suppress *Tcf7* expression in NK cells (Delconte et al., 2016), hence ID2 expression during the development of innate lymphocytes might contribute to the suppression of *Tcf7* expression as these cells mature.

1.5.5 RUNX

RUNX1 and RUNX3 play important roles in the development of several hematopoietic lineages. RUNX1 is necessary for the development of HSCs and lymphoid progenitors including LMPPs and CLPs (Wang et al., 1996; Wong et al., 2011; Satpathy et al., 2014). During T cell development,

RUNX1 is highly expressed in DN and DP thymocytes. RUNX1 is required for the repression of CD4 expression in DN thymocytes, efficient β- and positive selection processes and generation of NKT cells (Taniuchi et al., 2002; Egawa et al., 2005, 2007). RUNX3 is expressed by CD8 single positive thymocytes and represses CD4 and ThPOK expression in these cells (Setoguchi et al., 2008; Egawa et al., 2007; Egawa and Littman, 2008). During Th1 cell differentiation, RUNX3 cooperate with T-bet to repress *Il4* and activate *Ifng* transcription (Naoe et al., 2007; Djuretic et al., 2007; Kohu et al., 2009). RUNX3 also inhibits GATA-3 activity to promote Th1 cell differentiation. RUNX1 regulates RORγt and Th17 differentiation and maintains FOXP3 expression in Treg cells (Kitoh et al., 2009; Zhang et al., 2008).

NK cell-specific deletion of RUNX3 or CBF-β (*Cbfb*, the common obligatory partner of RUNX proteins) results in their great reduction and immature phenotype with low expression of Eomes, Ly49, DX5 and inefficient IFNγ production (Levanon et al., 2014; Ebihara et al., 2015). ILCPs highly express RUNX1, RUNX3 and CBF-β (Ebihara et al., 2015; Tachibana et al., 2011). Hematopoietic cell specific deletion of RUNX3 results in the accumulation of $Lin^{-}CD127^{+}GATA\text{-}3^{int}ROR\gamma t^{-}$ cells in the intestinal lamina propria (tissue resident ILC1 and ILC3 progenitors termed ILCLN cells), reductions of ILC1s and absence of ILC3s. RUNX3 is involved in the regulation of RORγt and AhR in ILC3s. In conclusion, RUNX3 is necessary for the differentiation of ILCLN cells into ILC1s and ILC3s, whereas it is dispensable for ILC2 development.

1.5.6 GATA-3

GATA-3 is a double zinc-finger transcription factor upregulated early during T cell development by Notch and TCF-1 signaling (Obaldia and Bhandoola, 2015). It is required for the establishment of the early thymic T cell progenitors, induction of BCL11b expression, T cell lineage

specification and commitment (Ting et al., 1996; Hosoya et al., 2009; García-Ojeda et al., 2013), β-selection and CD4 single positive thymocyte development (Henderson et al., 1994; Pai et al., 2003; Hernández-Hoyos et al., 2003; Wang et al., 2008). GATA-3 is required for the differentiation of Th2 cells, as well as Th2 cell maintenance and cytokine production (Zhu et al., 2004; Pai et al., 2004). TCR and cytokine stimulation promote GATA-3 expression in CD8 T cells to maintain their optimal expression of CD127, maintenance and proliferation (Wang et al., 2013).

GATA-3 is important at multiple stages during ILC development. EILPs express intermediate levels of GATA-3 and are partially dependent on it for their establishment (Harly et al., 2017). CHILPs also express intermediate levels of GATA-3 (Klose et al., 2014). ILCPs are GATA-3^{+} and are fully dependent on it for their development (Harly et al., 2017; Constantinides et al., 2014). GATA-3 deficiency in hematopoietic cells results in the loss of all T cells and ILCs (hence loss of LNs), but not NK cells (Yagi et al., 2014; Serafini et al., 2014). GATA-3 might also be required for the maintenance and function of ILC1s (Klose et al., 2014; Samson et al., 2003). Among ILCs, ILC2Ps and mature ILC2s highly express GATA-3 and are not only dependent on GATA-3 for their development but also maintenance and IL-5 and IL-13 cytokine production (Hoyler et al., 2012; Mjösberg et al., 2012; Liang et al., 2012). GATA-3 expression by ILC2s is also necessary for the induction of cytokine receptors ST2, IL-25R and CD25 (Yang et al., 2013). GATA-3 is also required for the CD127 expression and hence maintenance as well as IL-22 production of ILC3s (Zhong et al., 2016).

1.5.7 RORα

Retinoic acid receptor related orphan nuclear receptor (ROR) family includes RORα (*Rorα*), RORβ (*Rorb*) and RORγ (*Rorc*) (Jetten, 2007, 2009). RORγt, expressed by ILC3s, Th17 cells and

double positive thymocytes, is the thymus specific isoform of RORγ. *Rorα* is widely expressed in the family of innate lymphocytes. EILPs, ILCPs (Constantinides et al., 2014; Harly et al., 2017), fetal ILCPs and LTi progenitors (Ishizuka et al., 2016) as well as human systemic ILC precursors express *Rorα* (Lim et al., 2017b). All mature ILCs highly express *Rorα* (Hoyler et al., 2012; Robinette et al., 2015).

Mice with homozygous staggerer mutation (Rorα$^{sg/sg}$) have a spontaneous deletion of the RORα DNA-binding domain (Jetten, 2007, 2009). RORα is highly expressed in brain, specifically the cerebellum and thalamus. The Rorα$^{sg/sg}$ mice have underdeveloped cerebellar cortex resulting in staggering gait and tremor. In addition, RORα deficiency results in the loss of BM ILC2Ps and mature ILC2 in different tissues (Halim et al., 2012b; Wong et al., 2012). RORα-deficient mice do not seem to have defects in the development of ILC3s. However, ILC3 production of IL-17 and IL-22 is compromised in these mice (Lo et al., 2016), which is consistent with the role of RORα in regulating Th17 cell cytokine production (Yang et al., 2008). A large-scale, genome-wide association study of asthmatic versus healthy individuals showed that *Rorα* single-nucleotide polymorphisms (SNPs) were associated with asthma (Moffatt et al., 2010). In this study, SNPs in *Il18r1*, *Il1rl1*, *Il33* and *Il13* genes were also shown to be associated with asthma. In addition, RORα-deficient mice fail to develop allergic asthma and are protected from fibrogenesis (Jaradat et al., 2006; Lo et al., 2016). Therefore, RORα seems to be widely expressed during ILC lineage development and functional maturation.

1.5.8 BCL11b and GFI1

The zinc finger transcription BCL11b is upregulated by Notch signaling, directly and indirectly via TCF-1 and GATA-3, in DN2 thymocytes (Liu et al., 2010). Thereafter, BCL11b is

continuously expressed by DP, SP thymocytes and mature T cells. BCL11b deficiency results in a block from DN2 to DN3 stage of development (Wakabayashi et al., 2003). BCL11b deficient DN2 thymocytes express the stem/progenitor, myeloid and innate lymphocyte lineage associated genes (*Nfil3*, *Id2* and *Il2rb*), which should be downregulated at this stage (Li et al., 2010a). BCL11b deficient thymic T cell progenitors develop into self-renewing NK like cells (Li et al., 2010b). Hence, BCL11b is critical for T lineage commitment. In addition, BCL11b is required in mature T cells to inhibit their access to NK cell fate (Li et al., 2010b). BCL11b binds to GATA3 to co-localize at important cis-regulatory elements in Th2 cells (Fang et al., 2018) and blocks non-Th2 alternative cell fates in these cells.

A fraction of CHILPs and ILCPs expresses BCL11b (Yu et al., 2016). $BCL11b^{+}$ CHILPs only generate ILC2s, whereas $BCL11b^{-}$ CHILPs generate a mixture of ILC lineages. About a half of the $BCL11b^{+}$ CHILPs express IL-25R, and are considered early ILC2 progenitors. BCL11b deficiency in hematopoietic cells, resulted in the loss of these $IL\text{-}25R^{+}BCL11b^{+}PD\text{-}1^{+}$ CHILPs, ILC2Ps and mature peripheral ILC2s. BCL11b is specifically required during ILC2 development (Walker et al., 2015; Yu et al., 2015; Califano et al., 2015; Spooner et al., 2013) to maintain their genetic and functional identity. BCL11b directly regulates the transcriptional repressor growth factor independent 1 (GFI-1) in order to upregulate GATA3 and ST2 and inhibit SOX4, IL-23R, RORγt, IL-17 and IL-22 expression in ILC2s. In addition, BCL11b directly represses AHR expression in ILC2s. Consequently, BCL11b deficiency results in acquiring an ILC3 phenotype and function by ILC2s. It is notable that GFI-1 expression during Th2 differentiation of naïve $CD4^{+}$ T cells also promotes Th2 and antagonizes Th17 polarization (Zhu et al., 2009).

1.6 Thesis Objectives

The overall goal of my doctoral thesis was to elucidate the developmental pathways and complexity of lung ILC2s in neonatal and adult periods. My specific objectives were to first elucidate the early developmental process that leads to the generation of these cells in the BM, and second to elucidate ILC2 lineage heterogeneity in adult and neonatal lungs. Breaking down ILC2 developmental pathways, complexity and the unique functions of the ILC2 subsets may provide further insights into how ILC2 subsets contribute to specific pathological conditions including allergy and asthma and assist in developing specific therapeutic interventions.

According to the classical model of lymphopoiesis, early ILC progenitors including EILPs, CHILPs and ILCPs and hence mature ILCs develop from CLPs. In addition, it has been shown that mature ILCs can be generated from CLPs (Possot et al., 2011; Hoyler et al., 2012; Wong et al., 2012; Klose et al., 2014; Constantinides et al., 2014). However, previous studies by us and others have shown that LMPPs are much more efficient than CLPs in generating ILC2Ps and mature ILC2s in transplantation assays (Yang et al. 2011; Halim et al. 2012). Our primary results, obtained during my MSc studies in Dr. Fumio Takei laboratory, suggested that a subset of BM LMPPs expressing CD127 (defined as $Lin^{-}Kit^{hi}Sca\text{-}1^{hi}Flt3^{+}CD127^{+}$) expression was more efficient than CLPs defined as $Lin^{-}Kit^{lo}Sca\text{-}1^{lo}Flt3^{+}CD127^{+}$) in generating T cells, CHILPs and ILCs (Ghaedi, 2015) in transplantation assays. In addition, we realized that, while CLPs were rare, the CD127 expressing LMPPs were the predominant lymphoid progenitors in the neonatal period during which the development of T cells and ILCs is highly active. Hence the first objective of this thesis (discussed in Chapter3) was to determine whether there are CLP-independent developmental pathways for ILC development during adulthood. In order to achieve this objective, we characterized various adult BM lymphoid progenitors and assessed their gene expression

profiles as well as their capacities to differentiate into adaptive and innate lymphocytes. Our results collectively favored a temporal model of lymphocyte development, within which LMPPs directly contribute to the generation of T cells and innate lymphocytes and this pathway being highly active during neonatal period (Ghaedi et al., 2016). On the other hand, the generation of B cells required the CLP stage of development and was active mainly during adulthood.

The results obtained by pursuing the first objective of my thesis, led us to realize that lung ILC2 population was a heterogeneous population and hence we decided to study the complexity of this lineage in adult and neonatal periods. In addition, we were wondering about how the pool of long-lived lung resident ILC2s are maintained and whether adult BM progenitors are actively contributing to the generation of ILCs in tissues. Hence, the second objective of this thesis (discussed in chapters 4 and 5) is to elucidate the complexity of the lung ILC2 population during adult (discussed in chapter 4) and neonatal period (discussed in chapter 5), the functional properties of ILC2 subsets, as well as their developmental relationship. We have identified ILC2 cells resembling BM ILC progenitors in adult and neonatal lungs that might contribute to the generation of ILC2s in the lung as well distinct ILC2 effector subsets during neonatal period, hence provided evidence for the complexity of the ILC2 lineage.

Chapter 2: Materials and Methods

2.1 Mice

C57BL/6 (B6), B6.Ly5SJL, NSG, RORα-YFP and B6.*Rag1*$^{-/-}$ mice were obtained and maintained in the British Columbia Cancer Research Centre animal facility, under specific-pathogen-free conditions. RORα-YFP mice were generated by crossing B6.*RORα-IRES-Cre* (Chou et al., 2013) mice with B6.129X1-*Gt(ROSA)26Sortm1(EYFP)Cos*/J. The use of these mice was approved by the animal committee of the University of British Columbia and in accordance with the guidelines of the Canadian Council on Animal Care. *Tcf7*^{EGFP} mice (Yang et al., 2015b) were kept in NIH animal facility and their use was approved by relevant NIH Animal Care and Use Committees. Adult mice of 4–10 weeks of age and 12-day-old neonatal mice of both sexes were used.

2.2 Rag-1/Red Mice

BMs from Rag-1/Red (CD45.2) and B6 (control) (CD45.2) mice were received from Dr. Paul Kincade's laboratory, Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center. The BM cells (5–8 ×10^6) from Rag-1/Red or B6 mice were transferred into lethally irradiated B6.Ly5SJL mice. Eight weeks later, these mice were used any further experiments.

2.3 RORα-YFP mice

A fraction of RORα-YFP mice had spontaneous YFP expression in all their cells. These mice were excluded, by checking their blood lecukocytes, from any further analysis. Also, these mice lacked the expression of NK1.1 on their NK cells, and hence I used Nkp46 in all my analyses instead.

2.4 Primary Leukocyte Preparation

Lung tissue was minced and fragments were digested in 5 ml DMEM containing collagenase IV (142.5 U/ml; Thermo Fisher, 17104019) and DNase I (118,050 U/ml, Sigma-Aldrich, 9003-98-9) for 25 minutes at 37°C on a shaker (150 rpm). The single cell suspension was filtered through a 70μm cell strainer and washed once with 5 ml DMEM containing 10% FBS and Penicillin-Streptomycin (10,000 U/ml). Leukocytes were enriched by centrifugation (10 minutes, 650×g) on a 36% Percoll gradient (Sigma-Aldrich, P4937) and red blood cells (RBC) were lysed by ammonium chloride solution. Single cell suspensions were prepared from small intestine in the same way.

For BM cells, tibia, femur and pelvic bones were crushed in 10 ml PBS containing 2% FBS and Penicillin-Streptomycin (10,000 U/ml) and the single-cell suspension was filtered through a 70μm cell strainer and washed once with 5 ml of the same media. This was followed by RBC lysis by ammonium chloride.

2.5 Antibodies and Flow Cytometry

Single cell suspensions were incubated with anti-mouse CD16/32 antibody (clone 2.4G2). eFluor 450-conjugated Lin-antibody cocktail contained anti-CD3 (145-2C11; RRID:AB_10735092), TCRαβ (H57-597; AB_11039532), TCRγδ (GL3; AB_2574071), NKp46 (29A1.4; AB_10557245), CD19 (1D3; AB_2734905), CD11b (M1/70; AB_1582236), CD11c (N418; AB_1548654), Ly-6C/G (Gr-1) (RB6-8C5; AB_1548788) and Ter119 (TER-119; AB_1518808). For BM, anti-CD45R/B220 (RA3-6B2; AB_1548761) was added to the Lin-cocktail. For intestine, anti-NKp46 was removed from the Lin-cocktail. For intracellular transcription factor staining,

PerCP-eFluor 710-anti-EOMES (Dan11mag; AB_10609215), Pecy7-anti-T-bet (4B10; AB_11042699), eFluor 660- or PE-anti-GATA-3 (TWAJ; AB_10596663 or AB_1963600), PE-anti-ROR gamma (t) (B2D; AB_10805392) and Foxp3/Transcription staining buffer set (Thermo Fisher; 00-5523-00) were used. The rest of the antibodies used in this study include Pecy7-anti-Ly-6A/E (Sca-1) (D7; AB_469668), APC-anti-CD117 (c-Kit) (2B8; AB_469431), eFluor 450-anti-Ly-6D (49-H4; AB_2574089), FITC- or eFluor 700-anti-CD45.1 (A20; AB_465058 or AB_493733), eFluor 450-anti-NK1.1 (PK136; AB_2043877), V500-anti-CD45 (30-F11; AB_10697046), APC-anti-LPAM-1 (Integrin α4β7) (DATK32; AB_10730607), PerCP-eFluor 710- or APC-anti-IL-33R (ST2) (RMST2-2; AB_2573883 or AB_2573301), FITC-anti-T1/ST2 (DJ8; AB_947549), PerCP-eFluor 710-anti-CD218a (IL-18Ra) (P3TUNYA; AB_2573764), Brilliant Violet 605-anti-CD90.2 (Thy1.2) (53-2.1; AB_11203724), Alexa Fluor 700- or PE or Pecy7-anti-CD127 (A7R34; AB_657611 or AB_465844 or AB_469649), Brilliant Violet 711-anti-KLRG1 (2F1; AB_2738542) and PE-anti CD278 (ICOS) (7E.17G9; AB_466273). For the detection of YFP along with intracellular transcription factors from RORα-YFP mouse tissues, single cell suspensions were stained for cell surface markers and viability dye (Thermo Fisher, 65-0865-18), followed by a pre-fixation step with 0.5% paraformaldehyde, washed with 5 ml PBS containing 2% FBS, and stained for the intra-nuclear transcription factors per manufacturer's instructions. Flow cytometry analysis was performed on a BD Fortessa flow cytometer and FACSDiva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo 8.7 (Tree Star).

2.6 RNA Isolation and Microarray Gene Expression Analysis

RNA from purified progenitors (LMPP-, LMPP+, Ly6D^{-} CLP andLy6D^{+} CLP) was extracted using Trizol (Thermo Fisher). Agilent Bioanalyzer 2100, RNA amplification, and microarray services were performed by Sick Kids, The Centre for Applied Genomics. Hybridization was carried out on the Affymetrix Mouse GeneChip ST 2.0. All data analysis was performed with FlexArray 1.6.3 (Genome Quebec).

2.7 Single Cell mRNA Sequencing (scRNA-seq)

Adult and neonatal (12-day-old) RORα-YFP mouse lungs were processed and single cell suspensions were stained with viability dye (Thermo Fisher, 65-0865-18), anti-CD45 and Lin- cocktail antibodies as above. Viable CD45$^{lo/+}$Lin^{lo}YFP^{-} and YFP^{+} cells were purified at 2 to 1 ratio. For the neonatal sample, FITC-anti-CD8 (53-6.7; RRID:AB_464915) was added as a Lin antibody. The Purified Cells were loaded on the Chromium Single Cell Controller (10X Genomics) and libraries were prepared using the Chromium Single Cell 3′ Reagents Kits (v2 chemistry; 10X Genomics), according to the manufacturer's protocol. The obtained libraries were sequenced on a NextSeq 500 (Illumina). Cell Ranger software (v2.0.1; 10X Genomics) was used to perform demultiplexing, alignment, counting as well as initial clustering, and differential gene expression analysis. The reads were aligned to the mm10 genome, with the genome re-annotated to include YFP, TCR and immunoglobulin genes. ScRNA-seq analysis was performed using Seurat package (Butler et al., 2018) (version 2.1.0) in R (version 3.4.2) (R core team, 2017). The data were normalized and scaled to remove unwanted sources of variation according to the Seurat package criteria. The Seurat's RunPCA function was used to perform linear dimensional reduction and the first 19 principle components (PC) were used for further analysis. For the analysis of only ILC2s,

15 PCs were used for further analysis. Seurat's FindClusters and RunTSNE functions were used to identify the cell clusters and visualize them. A range of resolutions were tested, and a resolution of 0.6 was used. Before unsupervised clustering of ILC2 barcodes, B cells expressing *Cd79a*, *Cd79b*, *Ms4a1*, *Ebf1* or *Pax5*, NK cells expressing *Gzma*, *Gzmb*, *Eomes*, *Ncr1* or *Prf1*, stromal cells expressing *Scgb3a1*, T cells co-expressing any TCR genes and *Cd4*, *Cd8a* or *Cd5*, and NKT cells co-expressing any TCR genes and *Klrk1* were filtered out. Seurat's FindMarkers function was used to find differentially expressed genes among clusters/subsets. The FindMarkers functions were performed two times for adult and neonatal ILC2 subsets, once with only assessing genes that are present in at least 20% of the cells in either of the subsets. The second time this criterion was removed and all the genes that were differentially expressed between subsets were determined. Wilcoxon rank-sum tests were used to determine the significance of the differential expression. Single cell trajectory was constructed using the Monocle package (Trapnell et al., 2014) (version 2.6.1) in R. State 1 was manually selected as the root of the neonatal ILC2 differentiation trajectory based on the similar gene expression profile of this state to BM ILC progenitors (Yang et al., 2015b; Constantinides et al., 2014; Seehus et al., 2015a).

2.8 BM Transplantation

B6.Ly5SJL (CD45.1) mice were lethally irradiated (two doses of 6 Gy with 4-hr wait in between or 10 Gy in one dose) and intravenously injected with purified progenitors (CD45.2) and 10^5 helper BM cells from NSG mice (CD45.1). Drinking water of the irradiated mice was supplemented with HCl and ciprofloxacin for 4 weeks.

2.9 BrdU Administration

Neonatal mice received three daily intranasal injections of 0.4 mg of BrdU. Lungs were collected at indicated time points. Adult mice were given drinking water containing 0.8 mg/ml BrdU, which was prepared freshly every 2nd day until experimental endpoint. Staining for BrdU was done using a FITC BrdU Flow Kit (BD Biosciences).

2.10 Accession Code

The GEO accession numbers for the microarray and scRNA-seq data reported are GSE67034 and GSE122762, respectively.

2.11 Data and Materials Availability

ScRNA-seq data are deposited under the private GEO accession number GSE122762 and can be accessed using the token uxozmawypjwnfmr.

2.12 *In vivo* Stimulation

Mice were anesthetized by isofluorane inhalation first. Intranasal administrations of recombinant mouse IL-33 (0.25μg) (BioLegend, 580508) or papain (25μg) (Sigma-Aldrich, 76216) in 40 μl PBS were performed on days 0, 1, and 2. Lungs were collected and analyzed on day 5.

2.13 *In vitro* Culture

For each *in vitro* culture, lung cells were prepared from 12-20 mice. ILC2s and other cell populations were sorted by a BD FACSARIA III or FUSION. Purified adult ILC2s (500 cells) were cultured in 200 μl of RPMI1640 containing 10% FBS and Penicillin-Streptomycin (100

U/ml) with PMA (30 ng/ml) and ionomycin (500 ng/ml). Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, m60009rdpd) was used to determine the respective chemokines and cytokines. Purified neonatal ILC2s (1000 cells) were cultured in the same way with 1 or 10 ng/ml of IL-33 (BioLegend, 580508) and IL-7 (Thermo Fisher, PMC0071) each. IL-5 (RRID:AB_2574980), IL-13 (RRID:AB_2575028) and amphiregulin (R&D Systems, DY989) ELISA kits were used.

2.14 Statistics

Data were analyzed using GraphPad Prism 7 (GraphPad Software). Two-tailed Student's t or Mann-Whitney unpaired test was used to determine the statistical significance. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) were considered significant.

Chapter 3: Group 2 Innate Lymphoid Cells Develop via Common-Lymphoid-Progenitor-Independent Pathways

3.1 Introduction

ILCs are generally thought to develop from CLPs in the BM. However, our group and others have shown that LMPPs are more efficient than CLPs in generating ILCs and T cells in transplantation assays (Halim et al., 2012b; Yang et al., 2011; Allman et al., 2003). We realized that the neonatal period is a very important stage for lung ILC2 development (Ghaedi, 2015; Ghaedi et al., 2016; Steer et al., 2017). Within this period, LMPPs were the predominant lymphoid progenitors in the hematopoietic organs spleen and BM, whereas CLPs were rare. In addition, neonatal CLPs, unlike LMPPs, could not efficiently differentiate into T cells and ILC2s in transplantation assays. A small subset of LMPPs defined by their CD127 expression, which we termed LMPP+, was particularly efficient in differentiating into ILCs and T cells in transplantation assays (Ghaedi, 2015; Ghaedi et al., 2016). Therefore, neonatal T cells and ILC2s seemed to be mostly generated from LMPP+s. In this chapter of my thesis, we continued our previous studies to determine whether adult BM LMPP+s differentiate into ILCs via CLP-independent pathways. In addition, we investigated whether the large pool of ILC2s generated during the neonatal period become long-lived cells and persist into adulthood. These studies lead to the better understanding of the lymphoid progenitors involved in the ILC generation and how this process is regulated in the neonatal and adult periods.

3.2 Results

3.2.1 Lymphoid Progenitors in Adult Mouse BM Can Be Divided into Subsets with

We first divided adult mouse BM Lin^- cells into $Kit^{hi}Sca\text{-}1^{hi}$ and $Kit^{lo}Sca\text{-}1^{lo}$ subsets. The $Kit^{hi}Sca\text{-}1^{hi}$ cells included the conventional LMPPs defined as $Flt3^+CD127^-$, which we termed LMPP-s (Figure 3.1A). A small fraction of LMPPs was $CD127^+$ (Adolfsson et al., 2005; Luc et al., 2012), which we termed LMPP+s (Ghaedi, 2015; Ghaedi et al., 2016). The $cKit^{lo}Sca\text{-}1^{lo}$ subset included the $Flt3^+CD127^+$ CLPs (Karsunky et al., 2008; Kondo et al., 1997). In agreement with previous reports, we could divide CLPs into $Ly6D^-$ and $Ly6D^+$ subsets. LMPP-s were $Ly6D^-$ and only a small fraction of the LMPP+s was expressing Ly6D. In order to investigate whether LMPP-s, LMPP+s, $Ly6D^-$ and $Ly6D^+$ CLPs present distinct lymphoid progenitor subsets, they were purified from adult mouse C57BL/6 BM and compared by Affymetrix microarray gene expression analyses. All the populations expressed the key transcription factor genes required for early lymphocyte development (Rothenberg, 2014), including *Sfpi1* (PU.1), *Ikzf1* (IKAROS family zinc finger 1), *Tcf3* (E2A Immunoglobulin Enhancer Binding Factors E12/E47) and *Tcf12* (Transcription Factor 12) (Figure 3.1B). The expression of myeloid and B cell lineage associated genes showed a gradient of expression from LMPP-s to $Ly6D^+$ CLPs. The expression of myeloid lineage associated genes including *Cebpa*, *Ctsg* and *Prtn3* were highest in LMPP-s (Figure 3.1C), and showed a gradual decrease in LMPP+s, $Ly6D^-$ and $Ly6D^+$ CLPs. On the other hand, the expression of B cell lineage associated genes including *Ebf1*, *Pax5*, *Blk*, *Blnk*, *Vpreb1*, *Vpreb2* and *Cd79a* were lowest in LMPP-s (Figure 3.1D). The expression of these genes was slightly higher in LMPP+s than LMPP-s. These genes were expressed at higher levels in Ly6D+ CLPs than all other progenitors examined. The expression of T cell and ILC lineage associated genes were equally low among all the four populations (Figure 3.1E and F). The phenotype and gene

expression profiles of these progenitor populations suggested that that they might present sequential lymphoid progenitors that are committing into the lymphoid lineage by gradually losing their myeloid potentials, while they might have distinct potential for different lymphoid lineages.

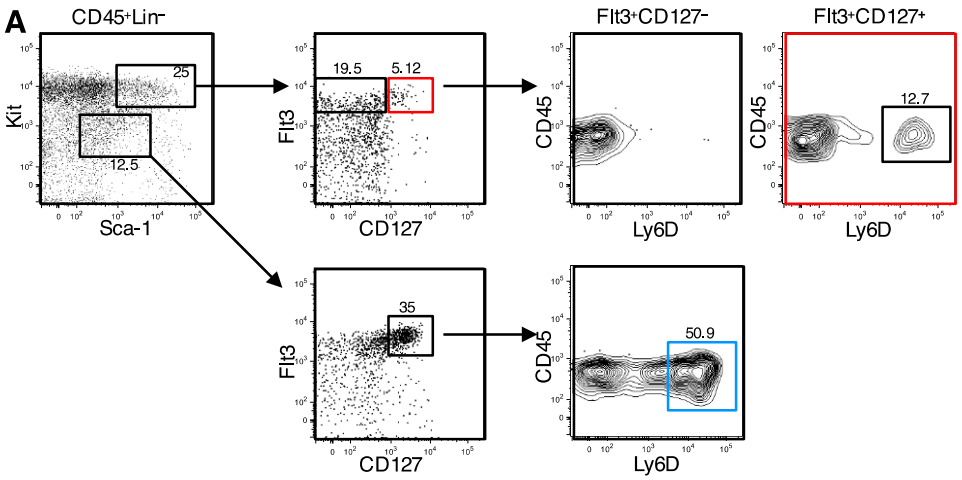


Figure 3.1 Adult

B

	LMPP-	LMPP+	Ly6D– CLP	Ly6D+ CLP
Sfpi1	11.01	10.56	10.32	10.15
Ikzf1	9.66	10.19	10.44	10.36
Tcf3	11.11	11.16	11.30	11.33
Tcf12	8.37	8.01	8.58	8.15

C

Myeloid-cell-lineage-associated

	LMPP-	LMPP+	Ly6D– CLP	Ly6D+ CLP
Cebpa	8.28	7.71	7.03	6.55
Hk3	10.37	8.46	6.60	3.48
Csf3r	8.08	7.47	6.80	4.83
Csf1r	9.33	7.21	8.39	6.10
Abca9	6.70	4.88	2.94	3.30
Clec7a	7.06	5.38	2.82	3.07
Fcgr2b	7.89	6.48	5.55	4.91
Ctsg	10.56	8.34	6.11	5.48
Fes	7.36	6.63	6.49	4.92
Prtn3	9.95	8.58	6.45	4.26
Ccl9	10.86	8.89	7.19	6.85

D

B-cell-lineage-associated

	LMPP-	LMPP+	Ly6D– CLP	Ly6D+ CLP
Il7r	4.25	9.49	9.81	10.06
Ly6d	3.42	8.23	5.62	11.41
Ebf1	5.42	7.84	7.54	10.24
Pax5	5.74	6.06	5.91	7.90
Foxo1	6.49	6.95	7.43	8.08
Rag1	3.41	8.30	8.21	11.11
Blk	3.82	8.54	8.41	10.40
Blnk	6.66	9.09	10.39	11.15
Vpreb1	6.45	6.88	7.82	10.09
Vpreb2	3.64	6.48	4.07	9.69
Cd79a	4.58	4.38	5.71	7.51

E

T-cell-lineage-associated

	LMPP-	LMPP+	Ly6D– CLP	Ly6D+ CLP
Notch1	8.45	8.86	8.88	8.97
Hes1	6.04	8.41	8.49	8.88
Lef1	6.69	6.44	6.48	8.11
Ccr9	4.88	6.26	6.21	6.09

F

ILC-lineage-associated

	LMPP-	LMPP+	Ly6D– CLPs	Ly6D+ CLP
Nfil3	6.23	6.08	6.85	6.24
Tcf7	5.36	6.10	7.25	5.63
Tox	6.49	7.22	8.32	7.07
Id2	6.96	7.10	6.25	7.20
Zbtb16	5.06	5.52	5.74	5.64
Gata3	4.82	5.20	5.48	5.46
Rora	5.30	5.02	4.96	5.20
Bcl11b	5.14	5.30	5.15	5.40
Tbx21	5.50	5.88	5.42	5.88
Eomes	4.90	4.23	5.47	4.80
Rorc	3.46	3.35	3.28	3.74
Itga4	5.93	6.08	6.77	6.23
Itgb7	5.28	5.51	5.52	5.53

BM Lymphoid Progenitors Are Divided into Four Subsets.

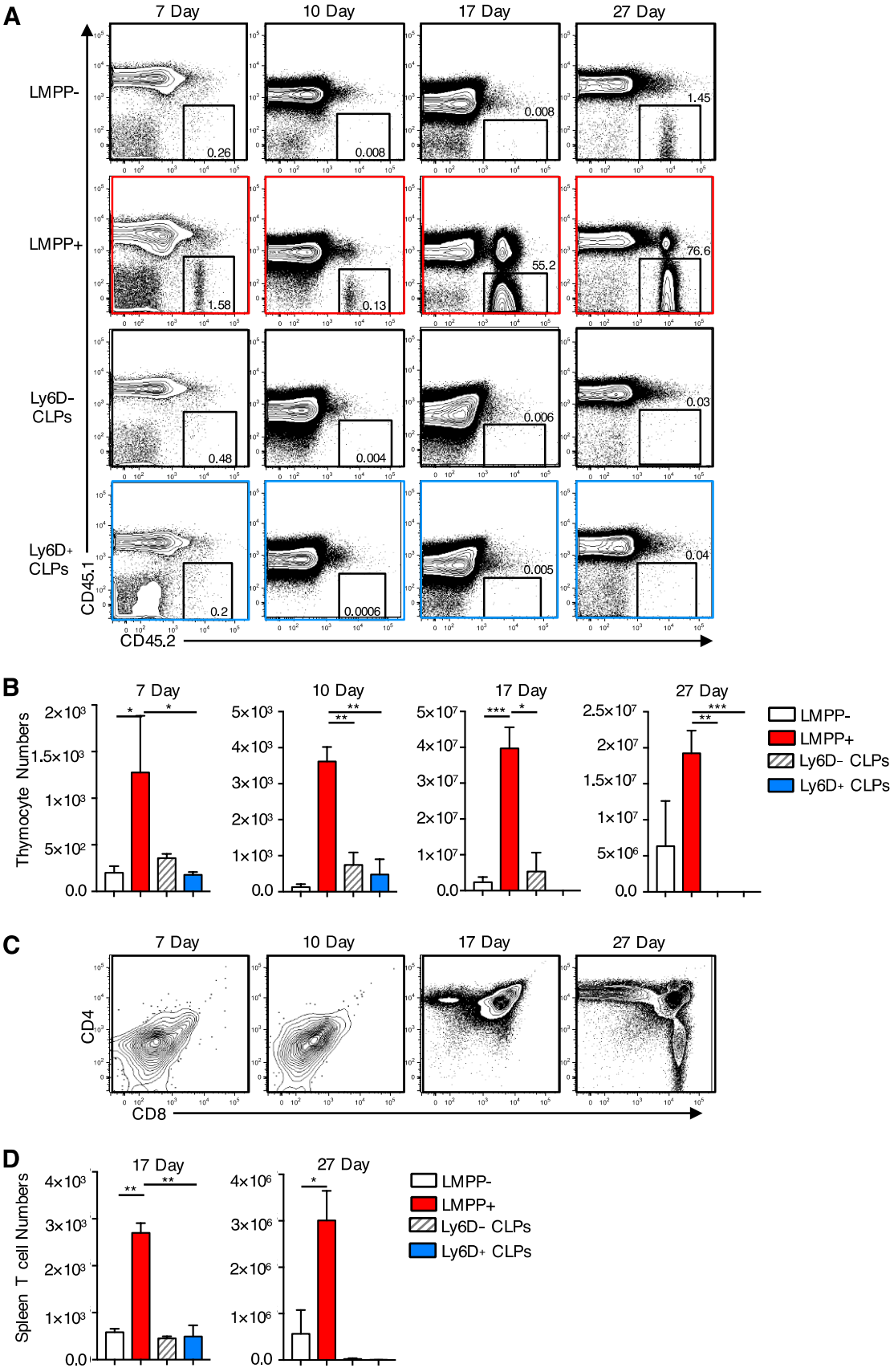
(A) Adult B6 mouse BM $CD45^+Lin^-$ cells were gated for $Kit^{hi}Sca\text{-}1^{hi}$ and $Kit^{lo}Sca\text{-}1^{lo}$. $Kit^{hi}Sca\text{-}1^{hi}$ cells were further divided into $Flt3^+CD127^-$ (LMPP-s) and $Flt3^+CD127^+$ (LMPP+s; red gate). $Kit^{lo}Sca\text{-}1^{lo}$ cells were gated for $Flt3^+CD127^+$ (CLPs). LMPP-s, LMPP+s and CLPs were analyzed for Ly6D expression. Numbers in plots indicate percentages of the gated cells. Data are representative of at least 3 independent experiments. LMPP-s (n = 3 replicates), LMPP+s (n = 3 replicates), $Ly6D^-$ (n = 2 replicates) and $Ly6D^+$ CLPs (n = 2 replicates) were purified from adult B6 mouse BM, and RNA was extracted from each progenitor population for Affymetrix microarray global gene expression analysis. Expression of the early lymphoid (B), myeloid (C), B cell (D), T cell (E) and ILC lineage associated genes (F) is shown. Numbers indicate normalized median values obtained by the Affymetrix Power Tools method. Lighter (yellow) and darker (blue) shading indicates higher and lower expression, respectively.

3.2.2 Adult BM LMPP+s Are Potent T cell Progenitors

To assess the differentiation capacities of LMPP-s, LMPP+s, $Ly6D^-$ and $Ly6D^+$ CLPs, these cells were purified from adult C57BL/6 mouse BM (CD45.2) and transplanted each into lethally irradiated congenic B6.Ly5SJL mice (CD45.1). The transplanted mice were analyzed at 7, 10, 17, and 27 days post-transplantation to determine the kinetics of lymphocyte development from each progenitor population. LMPP+s had the most rapid engraftment in the thymus (7 days) and generated much higher numbers of thymocytes than LMPP-s, $Ly6D^-$ CLPs and $Ly6D^+$ CLPs at all the time points post-transplantation (7, 10, 17, and 27 days) (Figure 3.2A and B). LMPP+-derived thymocytes increased in numbers from ~1500 on day 7 to ~ 4×10^7 on day 17. LMPP+-derived thymocytes gradually progressed from CD4 and CD8 double negative thymocytes on day 7 and 10 post-transplantation to double positive on day 17 and eventually single-positive on day 27 (Figure 3.2C). LMPP-s were slower than LMPP+s in the generation of thymocytes. Both $Ly6D^-$ and $Ly6D^+$ CLPs generated much less thymocytes than LMPP+s at all the time points post-transplantation. LMPP+-derived thymocyte numbers decreased from ~ 4×10^7 on day 17 to ~ 2×10^7 on day 27, suggesting that single positive T cells are leaving the thymus during this period. Hence, we analyzed donor-derived T cells in the spleen at 17 and 27 days post-transplantation of

the four progenitor populations. LMPP+-transplanted mice had significantly higher numbers of donor-derived T cells in the spleen than other subsets at both time points (Figure 3.2D).

In contrast to T cells, Ly6D$^+$ CLPs generated much higher numbers of spleen CD19$^+$B220$^+$ B cells than LMPP-s, LMPP+s and Ly6D$^-$ CLPs at 7 and 10 days post-transplantation (Figure 3.2E and F). LMPP progenitor subsets generated more B cells than CLP subsets at 17 and 27 days post-transplantation. LMPP+s rapidly generated NK cells in the spleen at 7 days post-transplantation (Figure 3.2G). More NK cells were generated from Ly6D$^-$ CLPs than other progenitor subsets at 10 days. At 17 and 27 days, more NK cells were generated from LMPP subsets than CLPs. In addition, myeloid cells (CD11b$^+$TCRαβ/γδ$^-$NK1.1$^-$) were generated in the spleen from LMPP-s at 7 days post-transplantation. Small numbers of myeloid cells were also generated from LMPP+s (Figure 3.2H).


A
7 Day
10 Day
17 Day
27 Day
LMPP-
LMPP+
Ly6D-
CLPs
Ly6D+
CLPs
CD45.1
CD45.2
0.26
0.008
0.008
1.45
1.58
0.13
55.2
76.6
0.48
0.004
0.006
0.03
0.2
0.0006
0.005
0.04
B
Thymocyte Numbers
LMPP-
LMPP+
Ly6D- CLPs
Ly6D+ CLPs
C
CD4
CD8
D
Spleen T cell Numbers

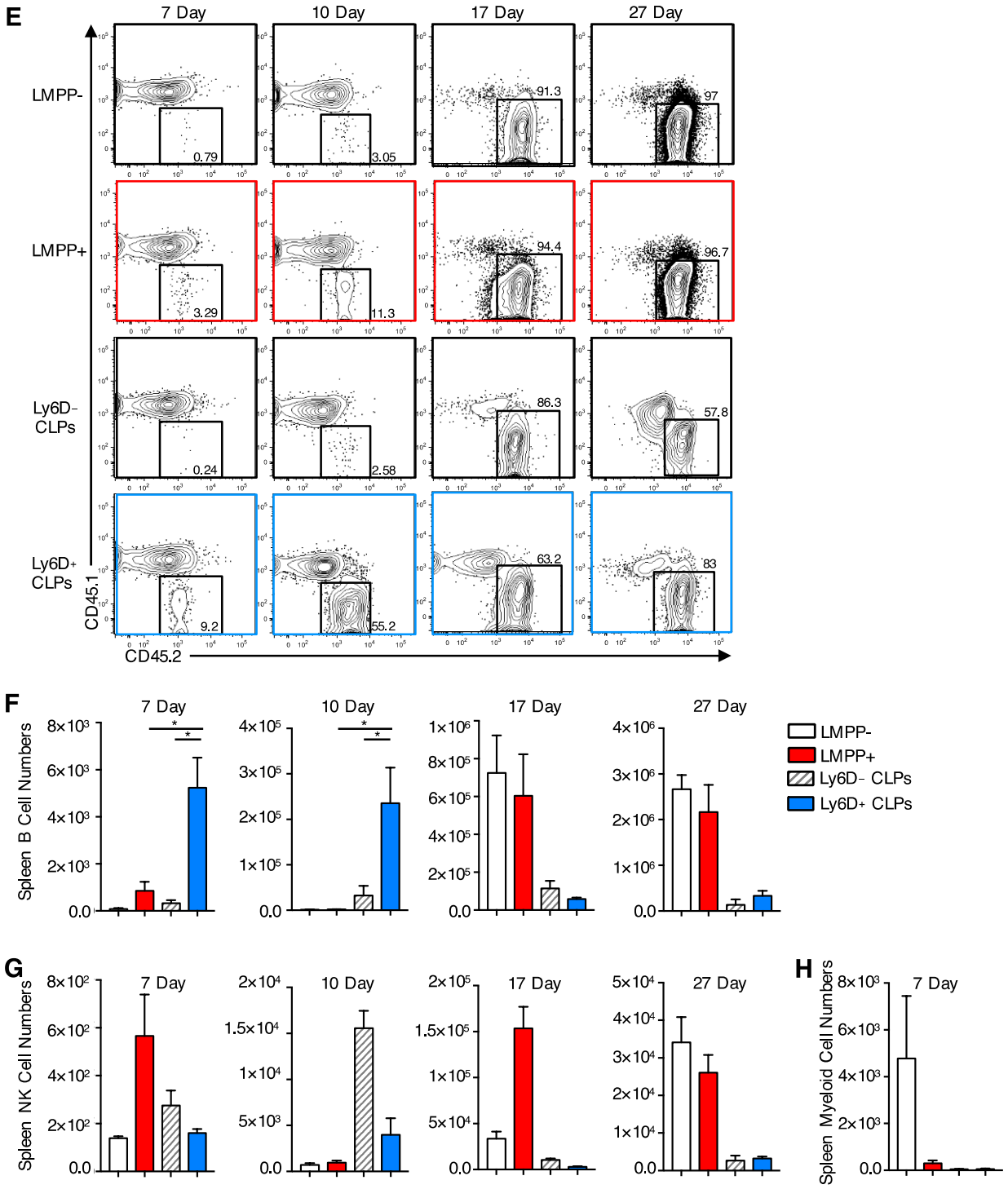


Figure 3.2 LMPP+s Are More Efficient than LMPP-s, Ly6D− and Ly6D+ CLPs at Generating T Cells

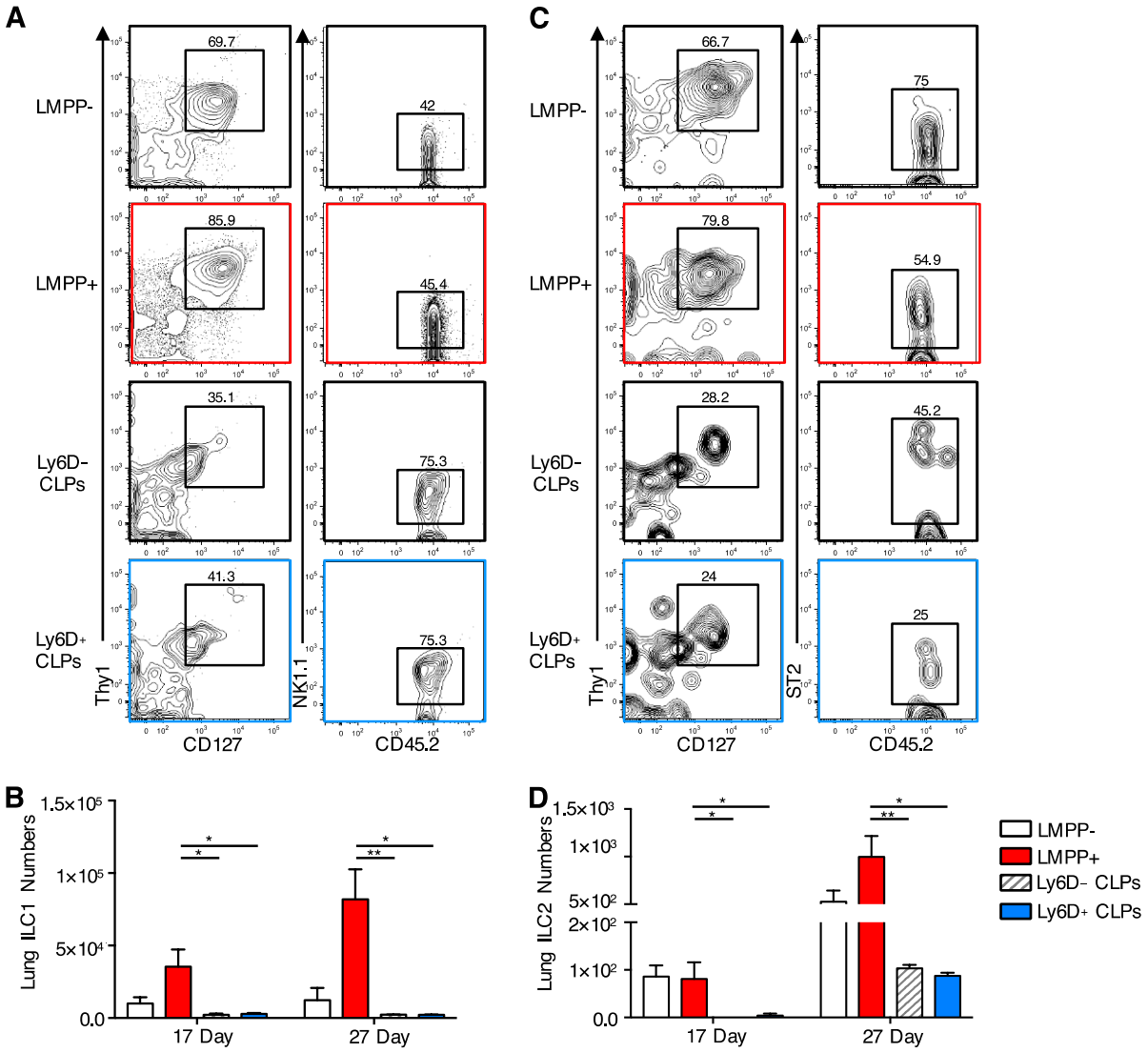
LMPP-s, LMPP+s, Ly6D$^-$ and Ly6D$^+$ CLPs were purified from adult C57BL/6 mouse BM (CD45.2+), and 2,000 cells of each progenitor population were intravenously injected along with 10^5 BM cells from NSG mice (CD45.1$^+$) into lethally irradiated congenic B6.Ly5SJL recipients (CD45.1$^+$). The tissues of the recipient mice were analyzed by flow cytometry at 7, 10, 17, and 27 days post-transplantation. (A) The cells in the thymuses of the recipient mice were

analyzed by flow cytometry. Live donor-derived cells ($CD45.1^{-}CD45.2^{+}$) were gated. (B) Absolute numbers of donor-derived thymocytes are shown in bar graphs. (C) Live donor-derived cells ($CD45.1^{-}CD45.2^{+}$) from the LMPP+ transplanted mice were further analyzed for CD4 and CD8 expression. (D) Absolute numbers of donor-derived T cells ($TCR\alpha\beta^{+}NK1.1^{-}$) in the spleen of the transplanted mice at 17 and 27 days post-transplantation are shown in bar graphs. (E) B cells ($CD19^{+}B220^{+}$) in the spleens of the recipient mice were gated for derived cells ($CD45.1^{-}CD45.2^{+}$). (F) Absolute numbers of donor-derived B cells are shown in bar graphs. (G) Absolute numbers of donor-derived NK cells ($NK1.1^{+}TCR\alpha\beta^{-}$) in the spleen are shown in bar graphs. (H) Absolute numbers of donor-derived myeloid cells ($CD11b^{+}TCR\alpha\beta/\gamma\delta^{-}NK1.1^{-}$) in the spleen are shown in bar graphs. Two-tailed Student's t or Mann-Whitney unpaired test was used to determine the statistical significance. Mean ± SEM; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) were considered significant. Data are representative of at least three experiments with four mice in each condition.

3.2.3 Adult BM LMPP+s Are Potent Progenitors for ILC1s, ILC2s and ILC3s

Donor-derived ILC1 ($NK1.1^{+}CD127^{+}Thy1^{+}$) and ILC2s ($Lin^{-}CD127^{+}Thy1^{+}ST2^{+}$) could be detected in the lungs of the recipient mice at 17 and 27 days post-transplantation. These cells were undetectable at the earlier time points. The number of LMPP+-derived ILC1s was considerably higher at 17 and 27 days post-transplantation compared to the very few generated from other progenitor subsets (Figure 3.3A and B). In addition, LMPP+s generated significantly higher numbers of ILC2s compared to $Ly6D^{-}$ and $Ly6D^{+}$ CLPs at both time points (Figure 3.3C and D). Intestinal ILC generation was slow compared to lung and intestines were analyzed at 7 and 10 weeks post-transplantation. Significantly more intestinal ILC2s and ILC3s were generated from LMPP+s than $Ly6D^{-}$ and $Ly6D^{+}$ CLPs (Figure 3.3E and F) at the different time points. Thus, LMPP-s, LMPP+s, $Ly6D^{-}$ and $Ly6D^{+}$ CLPs are all capable of differentiating into T, B, NK cells and ILCs. However, these lymphoid progenitor subsets are considerably different from each other in their kinetics of differentiation. LMPP+s are the most rapid and potent progenitors of T cells and ILCs. In contrast, much smaller numbers of T cells and ILCs are generated from $Ly6D^{-}$ or $Ly6D^{+}$ CLPs. On the other hand, $Ly6D^{+}$ CLPs are the most rapid and potent progenitors to B cells at the earlier time points post-transplantation. LMPP-s, LMPP+s and $Ly6D^{-}$ CLPs are all efficient NK cell progenitors to varying degrees depending on the time point analyzed. In addition to

lymphocytes, myeloid cells are generated from LMPP-s in the spleen at 7 days post-transplantation. LMPP+s also generated small number of myeloid cells at the same time point.



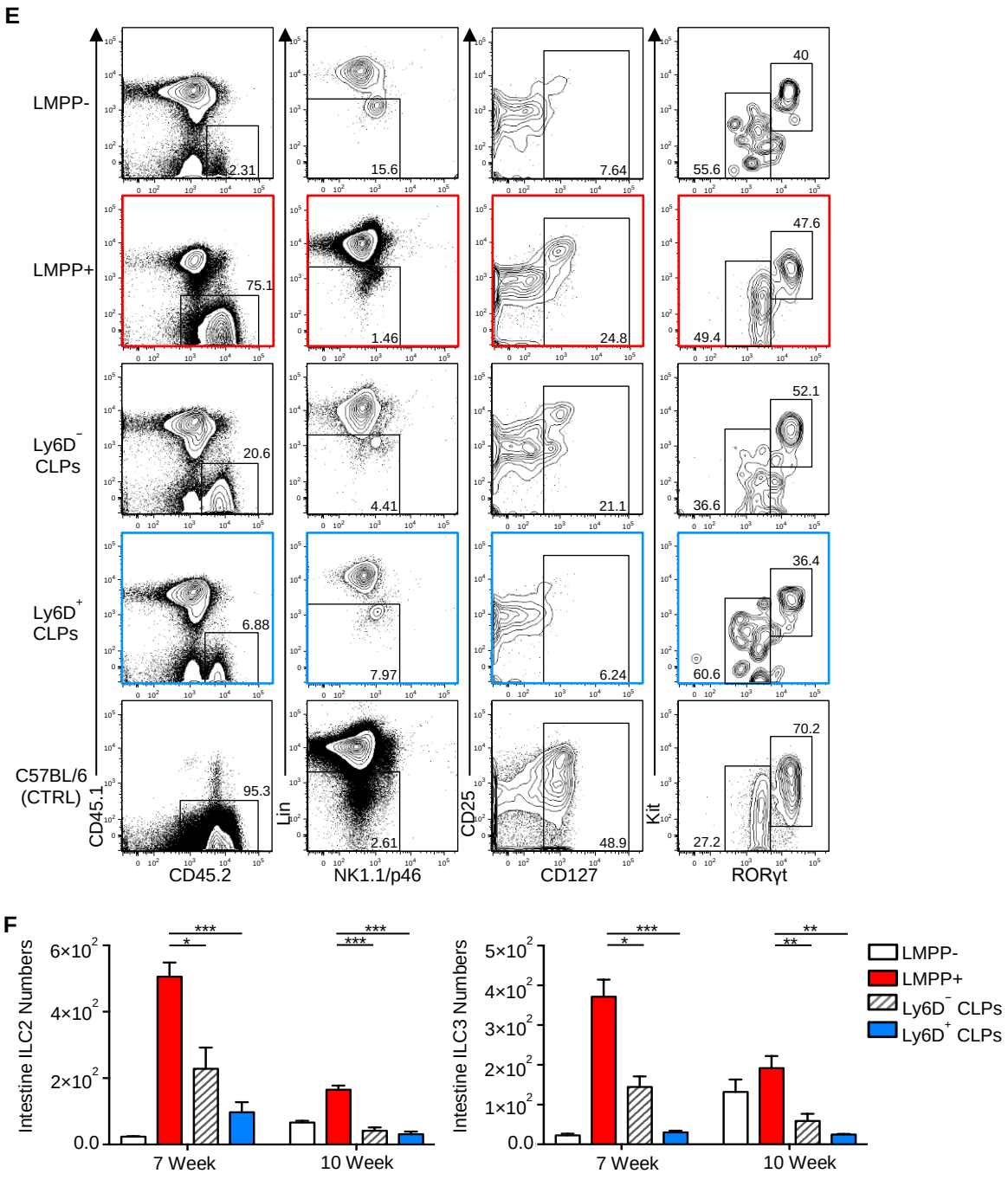


Figure 3.3 LMPP+s Are More Efficient than LMPP-s, Ly6D− and Ly6D+ CLPs at Generating ILCs

The lungs of the recipient mice were analyzed at 17 and 27 days post-transplantation for donor-derived ILC1s and ILC2s by flow cytometry. (A) The contour plots show ILC1 gating strategy at 27 days post-transplantation. $CD45.1^{-}CD45.2^{+}$ cells were first gated and ILC1s were sequentially gated by $Thy1^{+}CD127^{+}NK1.1^{+}$. (B) Absolute numbers of donor-derived ILC1s are shown in bar graphs. (C) The contour plots show ILC2 gating strategy at 27 days post-transplantation. $CD45.1^{-}CD45.2^{+}$ cells were first gated and ILC2s were gated by $Thy1^{+}CD127^{+}ST2^{+}$. (D) Absolute numbers of donor-derived ILC2s are shown in bar graphs.

The intestines of the recipient mice were analyzed at 7 and 10 weeks post-transplantation for donor-derived ILC2s and ILC3s by flow cytometry. (E) $CD45.1^{-}CD45.2^{+}Lin^{-}CD127^{+}$ cells were sequentially gated and divided into

RORγt^{-}Kit^{lo} (ILC2s) and RORγt^{+}Kit^{+} (ILC3s). (F) Absolute numbers of donor-derived ILC2s (left) and ILC3s (left) at 7 and 10 weeks post-transplantation are shown in bar graphs.

3.2.4 RAG-1 Lineage Tracing

In our primary studies (Ghaedi, 2015; Ghaedi et al., 2016), we analyzed the RAG-1-Cre/Rosa26 tandem dimer red fluorescent protein (tdRFP) mice (RAG/Red), in which cells expressing RAG-1 during their development should be irreversibly labeled by tdRFP. Our analyses indicated that LMPP-s were RAG/Red^{-}, ~25% of LMPP+s were RAG/Red^{+}. Ly6D^{-} and Ly6D^{+} CLPs were ~40% and 60% RAG/Red^{+}, respectively. On the other hand, ~40% of adult lung ILC2s were RAG/Red^{+}. To further investigate the differentiation potential of adult BM lymphoid progenitors into ILC2 and T lineages, LMPP-s, RAG/Red^{-} LMPP+s, RAG/Red^{-} and RAG/Red^{+} CLPs were purified from adult RAG/Red mouse BM and transplanted each into lethally irradiated congenic B6.Ly5SJL mice. RAG/Red^{-} LMPP+ transplantation resulted in the most-efficient ILC2 generation in the lung (Figure 3.4A and B), whereas RAG/Red^{-} and RAG/Red^{+} CLPs generated very small numbers of ILC2s. RAG/Red^{-} LMPP+ also engrafted thymus and generated thymocytes much more efficiently than other progenitor populations (Figure 3.4C and D). These results further suggested that LMPP+s are the upstream lymphoid progenitors to ILC progenitors including EILPs, CHILPs and ILCPs as well as ETPs, hence their transplantation results in efficient ILC and T cell generation.

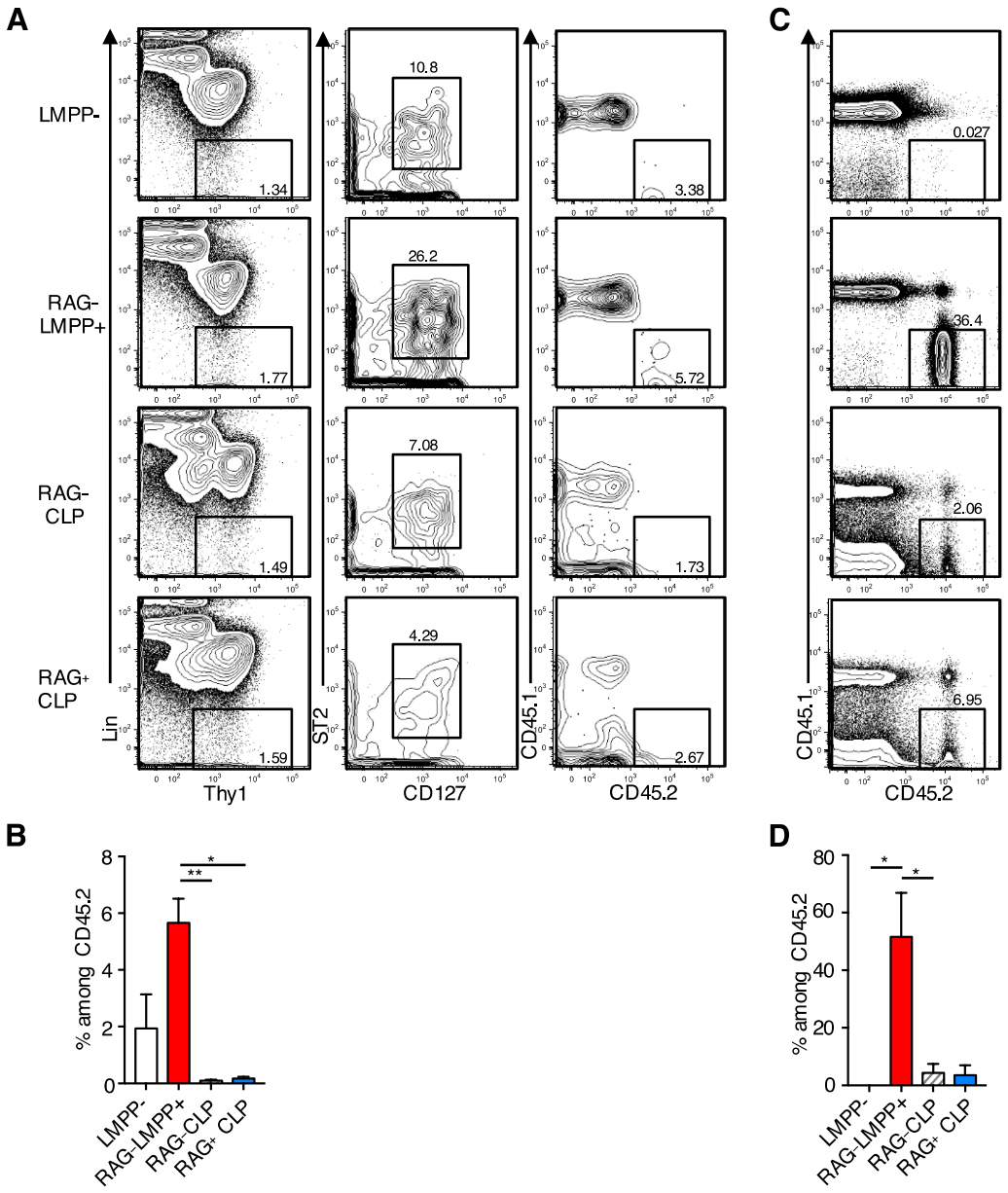


Figure 3.4 RAG-1 Lineage Tracking

BM cells (5–8 ×10^6) from Rag-1/Red (CD45.2) mice or normal B6 mice (control) (CD45.2) were transferred into lethally irradiated B6.Ly5SJL mice. Eight weeks later, Donor-derived LMPP-s (RAG/Red$^-$), RAG/Red$^-$ LMPP+s, RAG/Red$^-$ and Rag/Red$^+$ CLPs were purified from the Rag-1/Red transplanted mice and intravenously injected (2,000 cells per mouse) into lethally irradiated B6.Ly5SJL mice (CD45.1), along with 10^5 BM cells from NSG mice (CD45.1). (A) The lungs of the recipient mice were analyzed at 3 weeks post-transplantation for donor-derived ILC2s, sequentially gated by Lin$^-$Thy1$^+$ST2$^+$CD127$^+$CD45.1$^-$CD45.2$^+$. (B) The bar graph shows the absolute numbers of donor-derived lung ILC2s. (C) Thymocytes of the recipient mice were also analyzed for donor-derived cells (CD45.1$^-$CD45.2$^+$) in contour plots. (D) The bar graph shows the absolute numbers of donor-derived thymocytes.

3.2.5 Neonatal Lung ILC2s Persist into Adulthood

We established that adult LMPP+s are potent progenitors to T cells and ILCs. In addition, our primary results indicated that these cells are the predominant lymphoid progenitors in spleen and BM in the neonatal period, during which T cells and ILCs are actively developing (Ghaedi, 2015; Ghaedi et al., 2016). To determine the turnover rate of adult mouse lung ILC2s, they were labeled by bromodeoxyuridine (BrdU) administration in drinking water. Adult lung ILC2s slowly incorporated BrdU, but the percentages of BrdU-labeled ILC2s remained less than 25 (Figure 3.5A) up to 30 days. This was not due to inefficient delivery of BrdU to the lung, because the majority (over 75%) of lung ILC2s stimulated by intranasal papain injections were efficiently labeled by BrdU in drinking water (Figure 3.5B, left). ILC2 progenitors in the BM of naive mice were also labeled by BrdU (Figure 3.5B, right). Therefore, the large majority of lung ILC2s in naive adult mice seemed to be long-lived quiescent cells. To test whether ILC2s generated in the neonatal period persist and become quiescent ILC2s in adult lungs, neonatal mice received intranasal injections of BrdU at days 4, 6, and 9 after birth (Figure 3.5C, diagram). At day 10, a large majority of neonatal lung ILC2s was labeled by BrdU (Figure 3.5, left histogram). Six weeks after the BrdU injections, almost 30% of lung ILC2s were positive for BrdU, although the intensities of BrdU labeling significantly decreased (Figure 3.5C, right histogram). These results indicate that lung ILC2 development is highly active in the neonatal period, whereas the majority of adult lung ILC2s are quiescent with only a minor subset turning over. Furthermore, a sizable subset of adult lung ILC2s seem to be generated in the neonatal period.

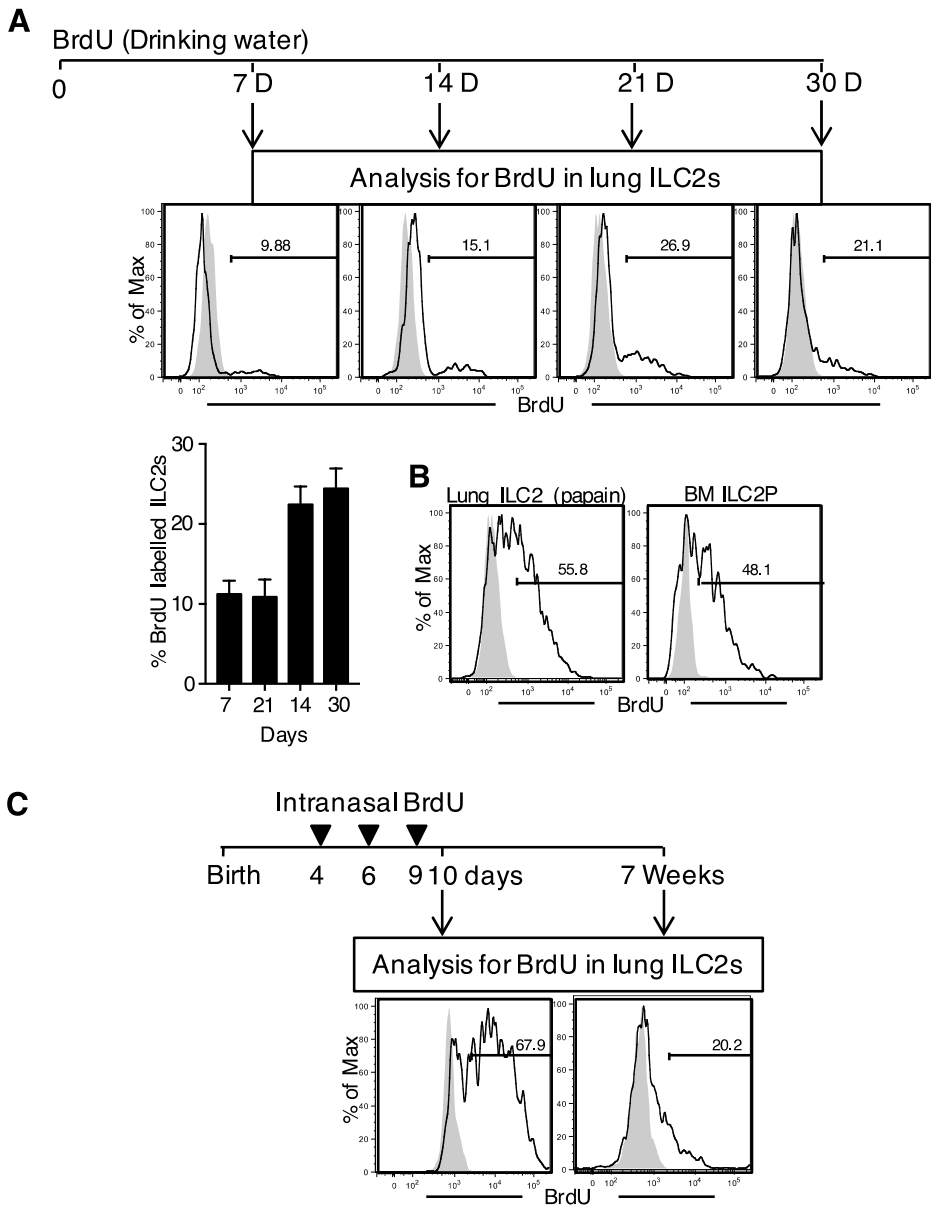


Figure 3.5 Neonatal Lung ILC2s Persist in Adult Mice
(A and B) Mice were given drinking water containing 0.8 mg/ml BrdU continuously. Lung ILC2s were analyzed (black) by flow cytometry for BrdU incorporation at the days indicated, after the start of BrdU treatment (A). Mice given plain drinking water were used as controls (gray). The percentage of BrdU-labeled lung ILC2s is also shown in a bar graph. BrdU-labeled lung ILC2s were analyzed from mice that received three consecutive intranasal doses of papain (15 mg, black) or PBS (control, gray) and analyzed 4 days later (B, left histogram). BrdU incorporation by BM ILCPs on day 14 for mice with BrdU (black) or plain drinking water (gray) is shown (B, right histogram). (C) Left diagram shows intranasal injections BrdU on days 4, 6, and 9. Lungs were collected at indicated time points. Right histograms show the staining for BrdU at indicated time points. The numbers indicate the percentages of cells in the gates. The data are representative of four replicates.

3.3 Discussion

HSCs differentiate into LMPPs that have a limited self-renewal capacity. LMPPs are thought to give rise to CLPs, which will further differentiate into pre-T, pre-B progenitors, NKP, and CHILP and eventually mature T, B, NK and ILCs. However, our results suggested that ILCs and T cells directly develop from a subset of LMPPs, termed LMPP+s, bypassing CLP stage of development. This pathway seemed particularly active during neonatal period, which is a critical stage of ILC and T cell lineage development (Ghaedi, 2015; Ghaedi et al., 2016). LMPP+s were considerably different from conventional LMPPs, termed LMPP-s in our study, in their gene expression profiles as well as their capacity to differentiate into lymphoid and myeloid cells. LMPP+s significantly differed from LMPP-s in the expression of myeloid and B-cell-lineage-associated genes. LMPP+s were more efficient in engrafting thymus and differentiating into thymocytes compared to LMPP-s. LMPP+s were also more efficient in differentiating into ILCs in the peripheral tissues than LMPP-s. On the other hand, LMPP-s were more efficient in generating myeloid cells. LMPP+s in our study are similar to ALPs (Inlay et al., 2009) in phenotype as well as lymphoid and myeloid lineage potential. It is notable that ALPs included both kit^{hi} and kit^{lo} cells, that likely corresponded to both LMPP+s and $Ly6D^{-}$ CLPs in our study. LMPP+s and $Ly6D^{-}$ CLPs were different from each other in the expression of myeloid and B cell lineage associated genes. Furthermore, LMPP+s were much more efficient than $Ly6D^{-}$ CLPs in thymic engraftment and differentiating into thymocytes as well as the generation of lung and intestinal ILCs. The kinetics analyses of the transplantation assays suggested that LMPP+s differentiated into T cells and ILCs, without first differentiating into $Ly6D^{-}$ CLPs. $Ly6D^{+}$ CLPs differentiated into splenic B cells much more rapidly and efficiently than LMPP-s, LMPP+s and $Ly6D^{+}$ CLPs. At later time points post-transplantation, more B cells are generated from LMPP+s than from Ly6D+ CLPs, which can be

explained by the development of more Ly6D+ CLPs from LMPP+s and subsequent generation of high numbers of B cells.

Normal adult mouse BM contains five times more CLPs than LMPP+s that possibly contribute to the ILC lineage in adult mice. However, our BrdU labeling experiments indicate that only a minor fraction of adult ILC2s seems to turn over. Furthermore, our previous studies (Ghaedi, 2015; Ghaedi et al., 2016) indicate that LMPP+s are the most abundant lymphoid progenitors in neonatal mouse BM and spleen when thymopoiesis and ILC development is most active. In addition, neonatal lung ILC2s persist into adulthood and become long-lived tissue resident cells. In addition to LMPP+s, CHILPs are also abundant in neonatal spleen (Ghaedi et al., 2016), which is in contrast to their small numbers and frequencies in adult BM (Klose et al., 2014; Gronke et al., 2016; Constantinides et al., 2014, 2015; Yang et al., 2015b). Therefore, it seems the development of ILCs from BM lymphoid progenitors in adults might be limited in steady state. The active pool of ILC progenitors during neonatal period might not only contribute to the generation of long-lived ILCs (Gasteiger et al., 2015), but also long-lasting pool of tissue resident ILC progenitors that can replenish ILC pool within tissues during adulthood. The existence of these tissue resident progenitors and ILC development within tissues are in agreement with the data showing clear differences in the tissue-specific proportional representation, phenotypes and functions of ILCs. In addition, our and another group analysis of adult RAG/Red mouse lung ILC2s (Ghaedi et al., 2016; Yang et al., 2011) indicated ILC2s are heterogeneous with regard to *Rag1* fate mapping. We showed that 40% of ILC2s are RAG/Red^{+}. These results along with BrdU labeling of neonatal lung ILC2s, which showed that about 30% of adult lung ILC2s are generated in the neonatal period provided evidence on lung ILC2 heterogeneity.

Our data suggest that some long-lived T cells and ILCs develop from LMPP+s bypassing the CLP stage at the neonatal stage and persist into the adulthood (Figure 3.6), whereas short-lived B cells are continuously produced from the prevalent BM Ly6D+ CLPs in adults.

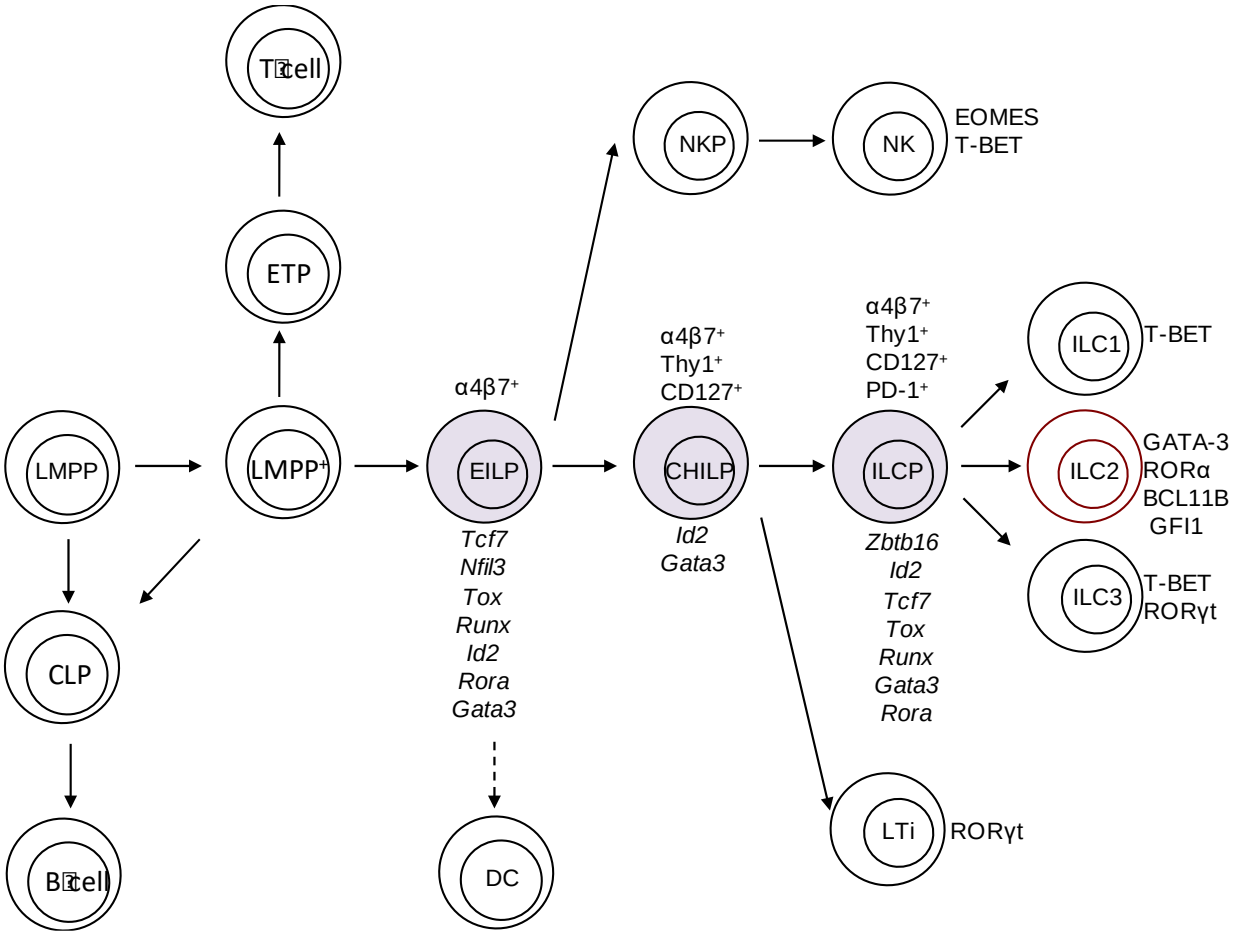


Figure 3.6 LMPP+s Are Upstream Lymphoid Progenitors to ILCs

Chapter 4: Single Cell Analysis of RORα Lineage Tracer Mice Revealed Heterogeneity of Adult Lung ILC2s

4.1 Introduction

Our results indicate that LMPP+s give rise to ILCs (Ghaedi et al., 2016). The BM common ILC precursors including EILPs (Yang et al., 2015b), CHILPs (Klose et al., 2014) and ILCPs (Constantinides et al., 2014) differentiate along the ILC lineage as they gradually upregulate the transcription factors TCF-1 (encoded by *Tcf7*), TOX (*Tox*), ID2 (*Id2*) and PLZF (*Zbtb16*) (Yu et al., 2016; Ishizuka et al., 2016; Harly et al., 2017).

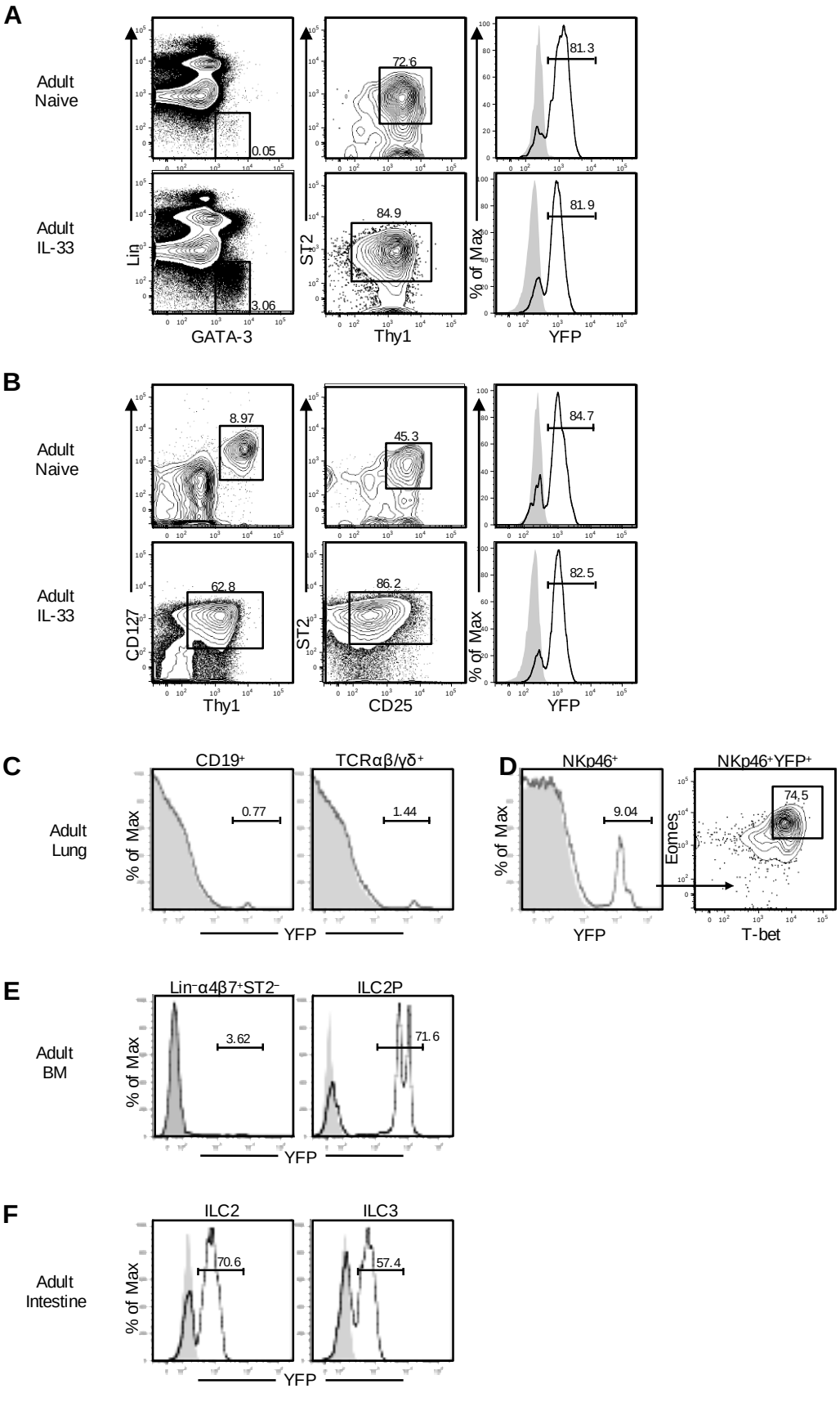
In mouse lungs, ILC2s can be defined as $Lin^{-}Thy1^{+}CD127^{+}ST2^{+}CD25^{+}$ that are $GATA3^{+}ROR\alpha^{+}$ (Halim et al., 2012b; Wong et al., 2012; Hoyler et al., 2012), and $Eomes^{-}T\text{-}bet^{-}ROR\gamma t^{-}$. Lung ILC2s play important roles in the development of allergic inflammation, and ILC2 numbers are elevated in the peripheral blood and sputum of asthma patients (Smith et al., 2016; Ying et al., 2016). ILC2s are involved in other lung diseases including COPD (Silver et al., 2016) and fibrosis (Hams et al., 2014). It is important to elucidate the complexity of the lung ILC2 population and the functional properties of ILC2 subsets, as well as their developmental relationship, so that proper subsets are targeted in therapeutic interventions. In order to study lung ILC2 complexity, we generated an ILC lineage tracer mouse model, based on the expression of the transcription factor RORα. *Rorα* is expressed in early ILC progenitors (Constantinides et al., 2014; Harly et al., 2017; Ishizuka et al., 2016; Lim et al., 2017a) and mature ILCs (Hoyler et al., 2012; Robinette et al., 2015; Halim et al., 2012b; Wong et al., 2012; Lo et al., 2016). Therefore, RORα lineage tracer mice enabled us to identify mature and immature ILC2s without relying on their expression of cell surface markers, specific cytokines or enzymes. To further adopt an

unbiased and comprehensive approach for studying ILC2 lineage complexity and heterogeneity, we analyzed all adult lung $CD45^{lo/+}Lin^{lo}$ cells by single cell RNA-sequencing and confirmed the results by flow cytometric and functional analyses. By this approach, we have identified a distinct cell population in adult lungs that resembles ILC progenitors in the BM.

4.2 Results

4.2.1 RORα Lineage Tracing Marks Innate Lymphocytes Including Lung ILC2s

We generated RORα lineage tracer mice by crossing *Rora-IRES-Cre* (Chou et al., 2013) and R26R-EYFP mice, which have a *loxP*-flanked transcription stop sequence followed by the gene encoding yellow fluorescent protein (YFP) in the ROSA26 locus. In these RORα-YFP mice, cells expressing *Rorα* during their development should be irreversibly labeled by YFP. As expected, most (>80%) conventional ILC2s, defined as $Lin^{-}GATA\text{-}3^{+}ST2^{+}Thy1^{+}$ (Figure 4.1A) or $Lin^{-}CD127^{+}Thy1^{+}ST2^{+}CD25^{+}$ (Figure 4.1B) were YFP^{+} in naïve adult mice. Intranasal IL-33 treatment resulted in the expansion of the YFP^{+} ILC2s. Less than 1% B ($CD19^{+}$) and 1.5% T cells ($TCR\alpha\beta/\gamma\delta^{+}$) in adult lungs expressed YFP (Figure 4.1C). About 9% of $TCR\alpha\beta/\gamma\delta^{-}NKp46^{+}$ lung cells were YFP^{+}, most of which were NK cells co-expressing Eomes and T-bet (Figure 4.1D). In the BM, only less than 4% of $Lin^{-}\alpha4\beta7^{+}ST2^{-}$ cells, which should include EILPs (Yang et al., 2015b), CHILPs (Klose et al., 2014) ILCPs (Constantinides et al., 2014) were YFP^{+} (Figure 4.1E). In contrast, the majority (>70%) of ILC2Ps ($Lin^{-}\alpha4\beta7^{+}CD127^{+}Thy1^{+}ST2^{+}$) (Halim et al., 2012b; Hoyler et al., 2012) were YFP^{+}. Small intestine ILC2s and ILC3s were also labeled by YFP, albeit to a lesser degree than lung ILC2s (Figure 4.1F).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Adult Naive
Adult IL-33
Lin
GATA-3
0.05
3.06
ST2
Thy1
72.6
84.9
% of Max
YFP
81.3
81.9
B
Adult Naive
Adult IL-33
CD127
Thy1
8.97
62.8
ST2
CD25
45.3
86.2
% of Max
YFP
84.7
82.5
C
Adult Lung
CD19+
TCRαβ/γδ+
% of Max
YFP
0.77
1.44
D
NKp46+
NKp46+YFP+
% of Max
YFP
9.04
Eomes
T-bet
74.5
E
Adult BM
Lin−α4β7+ST2−
ILC2P
% of Max
YFP
3.62
71.6
F
Adult Intestine
ILC2
ILC3
% of Max
YFP
70.6
57.4

Figure 4.1 YFP Expression in Lymphoid Populations of Lung, BM and Intestine of RORα-YFP Mice Shows the Expression of YFP by ILCs

(A) Lung ILC2s from naïve and IL-33 treated adult mice were sequentially gated by $CD45^{lo/+}Lin^{-}GATA\text{-}3^{+}ST2^{+}Thy1^{+}$ in contour plots, and their expression of YFP in RORα-YFP (black line) and B6 control (filled grey) mice is shown by histograms. **(B)** Lung ILC2s from naïve and IL-33 treated adult mice were sequentially gated by $CD45^{+}Lin^{-}CD127^{+}Thy1^{+}ST2^{+}CD25^{+}$ in contour plots, and their expression of YFP in RORα-YFP (black line) and B6 control (filled grey) mice is shown by histograms. **(C-D)** YFP expression by adult lung $CD19^{+}$ B cells and $TCR\alpha\beta/\gamma\delta^{+}$ T cells **(C)**, $TCR\alpha\beta/\gamma\delta^{-}NKp46^{+}$ ($YFP^{+}TCR\alpha\beta/\gamma\delta^{-}NKp46^{+}$ are analyzed for Eomes and T-bet expression) **(D)**. **(E)** BM $Lin^{-}\alpha4\beta7^{+}ST2^{-}$ and $Lin^{-}\alpha4\beta7^{+}CD127^{+}Thy1^{+}ST2^{+}$ ILC2Ps. **(F)** Intestinal $Lin^{-}ROR\gamma^{-}GATA\text{-}3^{+}$ ILC2s and $Lin^{-}ROR\gamma t^{+}GATA\text{-}3^{int}$ ILC3s in RORα-YFP (black line) and B6 control (filled grey) mice is shown by histograms.

4.2.2 RORα Lineage Tracing Marks $Lin^{-}ROR\gamma t^{-}GATA\text{-}3^{-}Thy1^{-}$ and $GATA\text{-}3^{lo}Thy1^{+}$ Cells in the Lung

The $Lin^{-}YFP^{+}$ cells in adult lungs, were $ROR\gamma t^{-}$ and included $GATA\text{-}3^{hi}Thy1^{+}$, $GATA\text{-}3^{lo}Thy1^{+}$ and $GATA\text{-}3^{-}Thy1^{-}$ cells (Figure 4.2). The $Thy1^{+}$ cells included $ST2^{+}CD25^{+}$ ILC2s and a small fraction of $ST2^{-}CD25^{-}$ cells. The $GATA\text{-}3^{-}Thy1^{-}$ cells were negative for the expression of all lymphoid-associated markers tested (data not shown). The $Lin^{-}YFP^{+}$ cells in the intestine included $GATA\text{-}3^{+}ROR\gamma t^{-}$ ILC2s and $GATA\text{-}3^{lo}ROR\gamma t^{+}$ ILC3s. Considering that ILC1s and NK cells were excluded from the $Lin^{-}YFP^{+}$ population in our analyses and $ROR\gamma t^{+}$ILC3s were scarce in the lung. Hence, these results suggested that $Lin^{-}YFP^{+}GATA\text{-}3^{lo}Thy1^{+}ST2^{-}CD25^{-}$ cells might be unconventional ILC2s.

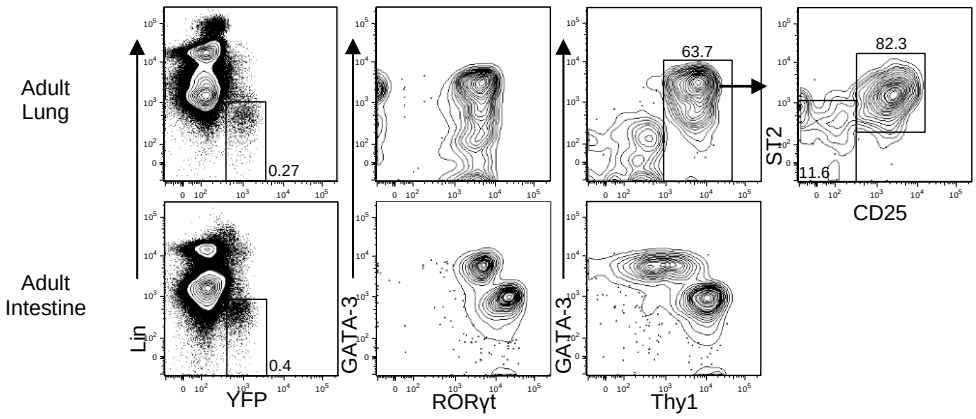
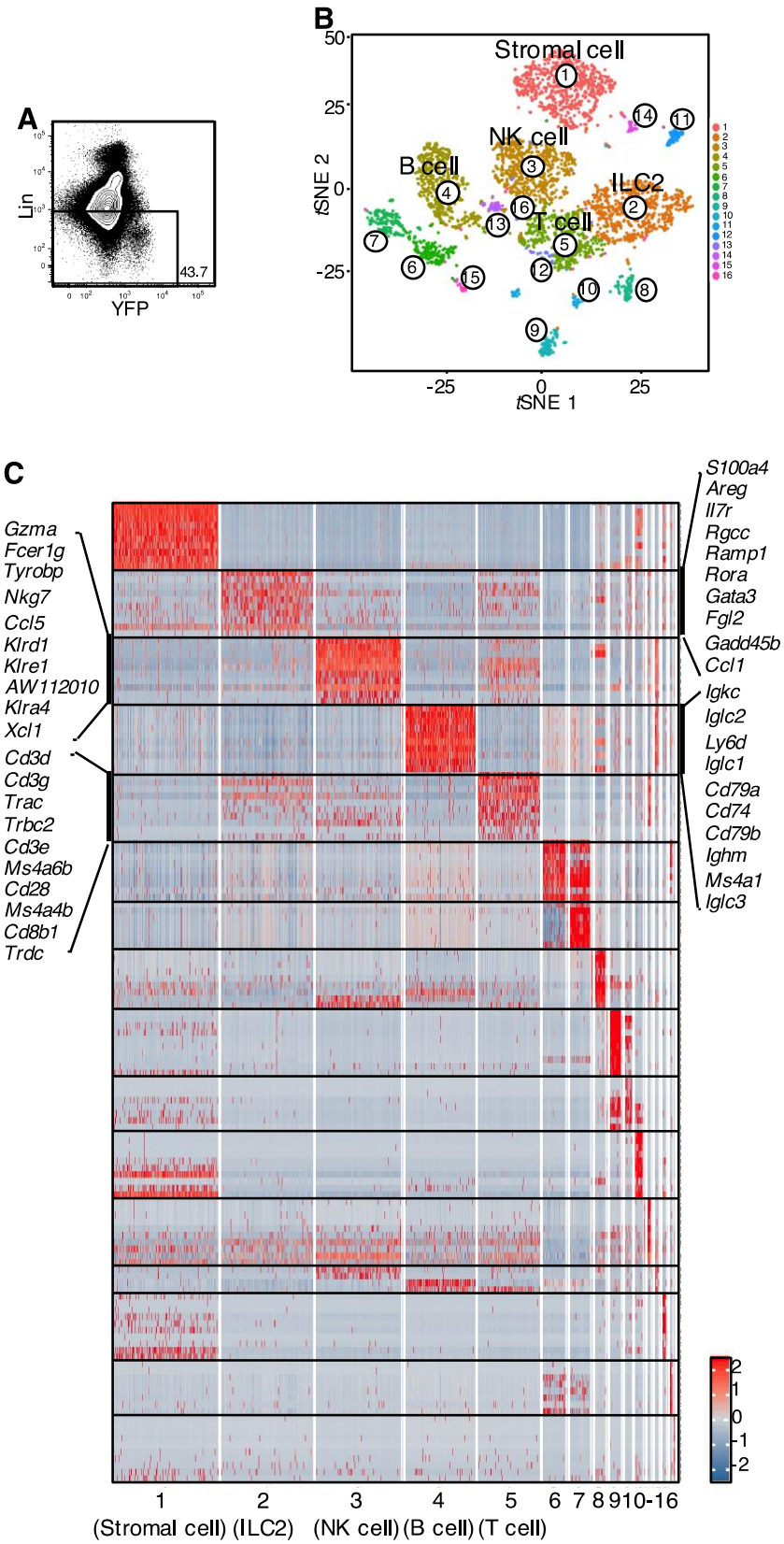


Figure 4.2 Flow Cytometric Analysis of RORα-YFP Mice Shows the Expression of YFP by Lin$^-$RORγt$^-$GATA-3$^-$Thy1$^-$ and GATA-3^{lo}Thy1$^+$ Cells in the Lung

The CD45$^{lo/+}$Lin$^-$YFP$^+$ cells from adult lungs and small intestine were gated and analyzed for the expression of GATA-3 and RORγt as well as GATA-3 and Thy1. The lung Lin$^-$YFP$^+$Thy1$^+$ cells are further analyzed for the expression of ST2 and CD25. Data are representative of at least 3 independent experiments with at least 3 mice per group in each experiment.

4.2.3 ScRNA-seq Analysis Identifies an ILC2 Cluster in Adult RORα-YFP Mouse Lungs

In order to fully assess the lung ILC2 heterogeneity, we analyzed all CD45$^{lo/+}$Lin^{lo} cells (Figure 4.3A) from adult (6-9 weeks old) RORα-YFP mouse lungs by droplet-based scRNA-seq. Unsupervised clustering of the cells in this dataset identified 16 distinct clusters (Figure 4.3B and C). ILC2 cluster was then identified by the expression of ILC2-associated genes including *Areg*, *Il7r*, *Rora* and *Gata3* (Figure 4.3C and Table 4.1). The ILC2 cluster was also evident on the *t*-SNE plot based on its high expression of *Rora*, *Gata3*, *Il1rl1*, *Areg* and *Arg1* (Figure 4.3D). Other clusters included stromal (*Gsn*, *Dcn*, *Clec3b*, *Apoe*, *Serping1* and *Inmt*), myeloid (*Siglech*, *Alox5ap*, *Ctsh* and *Irf8*), B (immunoglobulin genes, *Cd79a*, *Cd79b* and *Ms4a1*), NK (*Klra4*, *Klrd1*, *Klre1*, *Tyrobp*) and *Gzma*) and T cell (*Cd3e*, *Cd3d*, *Cd3g*, *Trac*, *Trbc2*, *Trdc* and *Cd28*) (Figure 4.3B and C, and Table 4.1). The *Yfp* was mostly expressed in the ILC2, NK and stromal cell clusters (Figure 4.3D). *Yfp*$^+$ NK and stromal cell clusters did not express *Rorα*, suggesting that these cells developed from *Rorα* positive progenitors.


A
Lin
YFP
43.7
B
Stromal cell
NK cell
B cell
ILC2
T cell
tSNE 2
tSNE 1
C
Gzma
Fcer1g
Tyrobp
Nkg7
Ccl5
Klrd1
Klre1
AW112010
Klra4
Xcl1
Cd3d
Cd3g
Trac
Trbc2
Cd3e
Ms4a6b
Cd28
Ms4a4b
Cd8b1
Trdc
S100a4
Areg
Il7r
Rgcc
Ramp1
Rora
Gata3
Fgl2
Gadd45b
Ccl1
Igkc
Iglc2
Ly6d
Iglc1
Cd79a
Cd74
Cd79b
Ighm
Ms4a1
Iglc3
1 2 3 4 5 6 7 8 9 10-16
(Stromal cell) (ILC2) (NK cell) (B cell) (T cell)

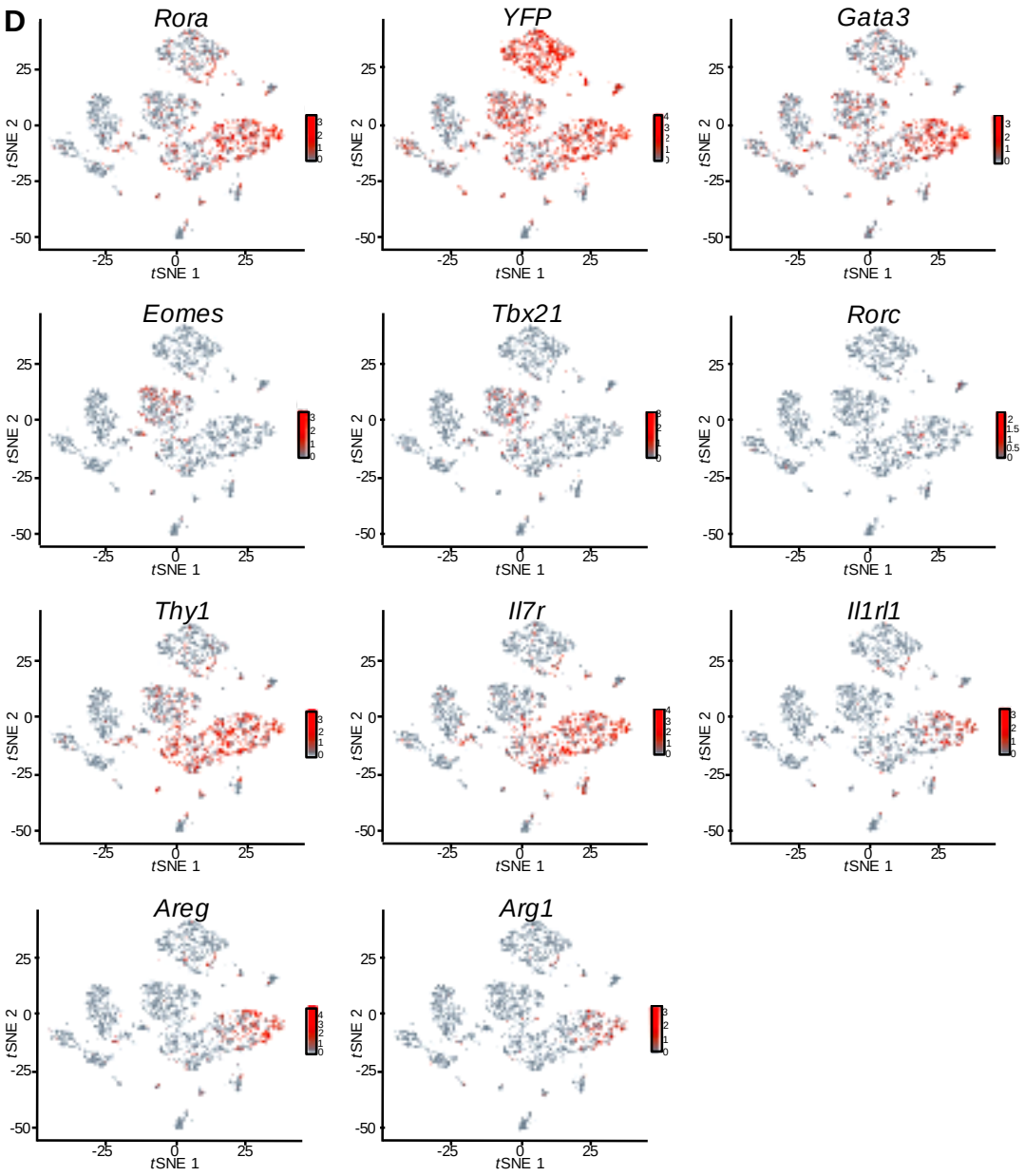


Figure 4.3 ScRNA-seq Analysis of Adult RORα-YFP Mouse Lungs Identifies an ILC2

(A) Gating strategy for the purification of Lin^{lo} cells from adult RORα-YFP mouse lungs for scRNA-seq analysis. **(B)** *t*-distributed stochastic neighbor embedding (*t*-SNE) plot shows distinct clusters within the 4,664 sequenced cells. **(C)** Heatmap of the differentially expressed genes by the clusters from **B**. Top 10 differentially expressed transcripts of the lymphoid lineages are shown. **(D)** *t*-SNE plots showing expression of the indicated individual genes.

Cluster 1	**Cluster** 2	**Cluster** 3	**Cluster** 4	**Cluster** 5	**Cluster** 6	**Cluster** 7	**Cluster** 8	**Cluster** 9	**Cluster** 10	**Cluster** 11	**Cluster** 12	**Cluster** 13	**Cluster** 14	**Cluster** 15	**Cluster** 16
Gsn	*S100a4*	*Gzma*	*Igkc*	*Cd3d*	*Cbr2*	*Scgb3a1*	*Cox6a2*	*Car4*	*Ccl21a*	*Upk3b*	*2810417H13Rik*	*Cd79a*	*Hhip*	*S100g*	*Hbb-bt*
Dcn	*Areg*	*Fcer1g*	*Iglc2*	*Cd3g*	*Hp*	*Bpifa1*	*Siglech*	*Cldn5*	*Mmrn1*	*Gpm6a*	*Birc5*	*Ly6d*	*Enpp2*	*Slc34a2*	*Alas2*
Clec3b	*Il7r*	*Tyrobp*	*Ly6d*	*Trac*	*Sftpb*	*Scgb3a2*	*Alox5ap*	*Ramp2*	*Lyve1*	*Msln*	*Ccnb2*	*Iglc2*	*Tagln*	*Pla2g1b*	*Hbb-bh1*
Apoe	*Rgcc*	*Nkg7*	*Iglc1*	*Trbc2*	*Scgb1c1*	*Reg3g*	*Ccr9*	*Emp2*	*Ptprb*	*Upk1b*	*Rrm2*	*Ms4a1*	*Mustn1*	*Ager*	*Hba-a2*
Serping1	*Ramp1*	*Ccl5*	*Cd79a*	*Cd3e*	*Sftpa1*	*Bpifb1*	*Cd300c*	*Egfl7*	*Cldn5*	*Cldn15*	*Ube2c*	*Fcer1g*	*Thbs1*	*Lyz1*	*Snca*
Inmt	*Rora*	*Klrd1*	*Cd74*	*Ms4a6b*	*Cyp2f2*	*Wfdc2*	*Lair1*	*Ecscr*	*Egfl7*	*Igfbp5*	*Hist1h2ap*	*Cd79b*	*Aspn*	*Cxcl15*	*Hba-a1*
Mgp	*Gata3*	*Klre1*	*Cd79b*	*Cd28*	*Wfdc2*	*Tff2*	*Mpeg1*	*Kdr*	*Gpihbp1*	*Nkain4*	*Top2a*	*Iglc1*	*Acta2*	*Chil1*	*Hbb-bs*
Serpinf1	*Ccr8*	*Klrk1*	*Ighm*	*Ms4a4b*	*Scgb3a2*	*Lypd2*	*Sh3bgr*	*Cyp4b1*	*Apold1*	*Sbsn*	*Stmn1*	*Ighm*	*Bmp5*	*Lyz2*	*Bpgm*
Cyr61	*Fgl2*	*AW112010*	*Ms4a1*	*Cd8b1*	*Sfta2*	*Cyp2f2*	*Smim5*	*Calcrl*	*Ramp2*	*Krt19*	*Cks1b*	*Igkc*	*Cyp2e1*	*Lcn2*	*Fam220a*
Id3	*Thy1*	*Klra4*	*Iglc3*	*Lat*	*Sftpd*	*Cxcl17*	*Cybb*	*Tmem100*	*Gng11*	*Clu*	*Smc2*	*Tyrobp*	*Grem2*	*Sfta2*	*Ube2l6*
Igfbp4	*Ccdc184*	*Klra7*	*Vpreb3*	*Lef1*	*Dcxr*	*Sult1d1*	*Pld4*	*Cd36*	*Aqp1*	*Cav1*	*Dut*	*Cd74*	*Serpine2*	*Sftpc*	*Isg20*
Col1a2	*Emb*	*Ctsw*	*Ebf1*	*Gm8369*	*Ldhb*	*Fxyd3*	*Rnase6*	*Cdh5*	*Sdpr*	*Igfbp6*	*Hmgn2*	*Ebf1*	*Igfbp5*	*Sftpd*	*Cabin1*
Fbln1	*Zcchc10*	*Klra1*	*Cd24a*	*Hcst*	*Gsta4*	*Gsto1*	*Plaur*	*Ednrb*	*Tm4sf1*	*Slpi*	*Hmgb2*	*Gzma*	*Adamts1*	*Sftpb*	*Cd3e*
Timp3	*Itgb7*	*Xcl1*	*Fcmr*	*Tcf7*	*Selenbp1*	*Cbr2*	*Bst2*	*Kitl*	*S100a16*	*Tm4sf1*	*Tuba1b*	*Dok3*	*P2ry14*	*Sftpa1*	*Mta2*
Pcolce2	*Ltb*	*Ncr1*	*Siglecg*	*Trdc*	*Gsta3*	*Krt18*	*Ctsh*	*Icam2*	*Prss23*	*Aqp1*	*Cenpa*	*Ccl3*	*Myl9*	*Bex2*	*Ppp1r12a*
Mfap5	*Furin*	*Ccl4*	*H2-Ab1*	*Lck*	*Scgb1a1*	*Gsta4*	*Tcf4*	*Acvrl1*	*Hpgd*	*Hspb1*	*H2afx*	*Ncr1*	*Lum*	*Fabp5*	*1810058I24Rik*
Cxcl12	*Gadd45b*	*Ccl3*	*H2-Eb1*	*Ikzf2*	*Retnla*	*Hp*	*Plac8*	*Ly6c1*	*Fxyd6*	*Csrp2*	*2700094K13Rik*	*Vpreb3*	*Egr1*	*Wfdc2*	*Mkrn1*
Sparc	*Nfkb1*	*Klra9*	*H2-Aa*	*Ccl5*	*Mgst1*	*Retnla*	*Irf8*	*Crip2*	*Ifitm3*	*Rarres2*	*H2afz*	*Spib*	*Ier3*	*Napsa*	*Sdha*
Pi16	*Tcrg-C4*	*Spry2*	*Cd72*	*Ly6c2*	*Lypd2*	*Mgst1*	*Tyrobp*	*Tspan13*	*Ier3*	*Aebp1*	*Tubb5*	*Ighd*	*Tppp3*	*Elovl1*	
Mt1	*Ccl1*	*Gzmb*	*Iglv1*	*Trbc1*	*Prdx6*	*Scgb1a1*	*Ccl4*	*Ly6a*	*Sepp1*	*Fxyd3*	*H2afv*	*Gm8369*	*Atf3*	*Npc2*	

Table 4.1 Top 20 Differentially Expressed Genes by the 16 Cellular Clusters in Figure 4.3B and C

The table shows the top 20 genes that are differentially expressed ($p \leq 0.01$) by each cluster in Figure 4.3B and C. The genes were selected by the FindMarkers function in the Seurat package.

4.2.4 ScRNA-seq Analysis Identifies Two ILC2 Subsets in Adult RORα-YFP Mouse Lungs

Further unsupervised clustering of the ILC2 cluster divided ILC2s into two subsets (1 and 2), each expressing a distinct set of genes (Figure 4.4A-C). Subset 1 highly expressed the conventional ILC2-associated genes including *Il1rl1* (ST2) (Halim et al., 2012a; b), *Tnfrsf18* (GITR) (Nagashima et al., 2018), *Areg* (amphiregulin) (Monticelli et al., 2015, 2011) and *Arg1* (arginase-1) (Monticelli et al., 2016). In contrast, subset 2 infrequently expressed *Il1rl1* and *Tnfrsf18* and instead had high expression of *Cd7* (CD7), *Cd2* (CD2), *Runx3* (Runx3), *Tcf7* (TCF-1) and *Il18r1* (IL-18rα). The two subsets equally expressed *Id2*, *Gata3*, *Rora*, *Il7r*, *Thy1* and *YFP* (Figure 4.4D).

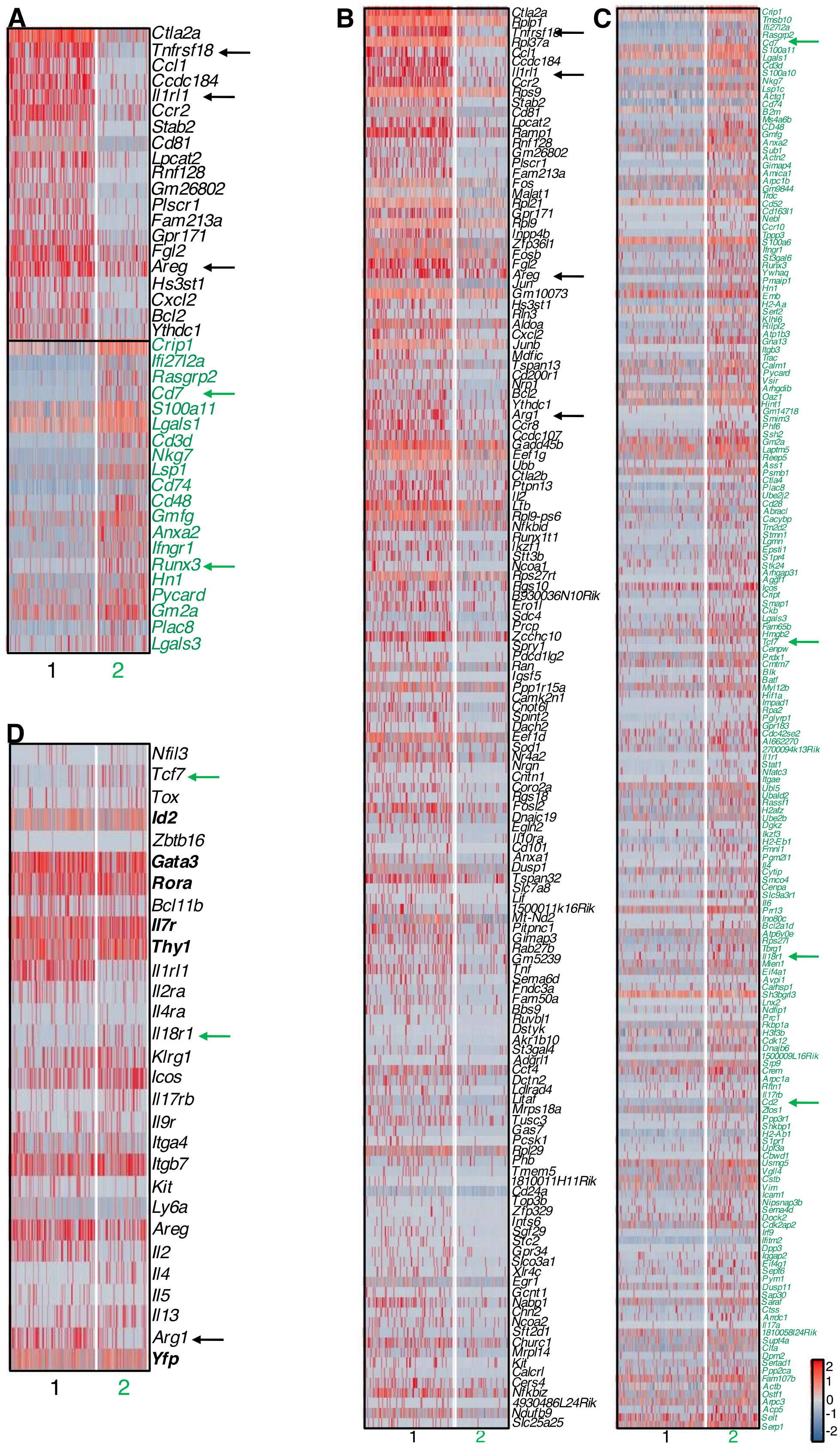

A
Ctla2a
Tnfrsf18
Ccl1
Ccdc184
Il1rl1
Ccr2
Stab2
Cd81
Lpcat2
Rnf128
Gm26802
Plscr1
Fam213a
Gpr171
Fgl2
Areg
Hs3st1
Cxcl2
Bcl2
Ythdc1
Crip1
Ifi27l2a
Rasgrp2
Cd7
S100a11
Lgals1
Cd3d
Nkg7
Lsp1
Cd74
Cd48
Gmfg
Anxa2
Ifngr1
Runx3
Hn1
Pycard
Gm2a
Plac8
Lgals3
1
2
B
C
D
Nfil3
Tcf7
Tox
Id2
Zbtb16
Gata3
Rora
Bcl11b
Il7r
Thy1
Il1rl1
Il2ra
Il4ra
Il18r1
Klrg1
Icos
Il17rb
Il9r
Itga4
Itgb7
Kit
Ly6a
Areg
Il2
Il4
Il5
Il13
Arg1
Yfp
1
2
2
1
0
-1
-2

Figure 4.4 ScRNA-seq Analysis of Adult RORα-YFP Mouse Lung ILC2s Identifies Two ILC2 Subsets with Distinct Gene Expression Profiles

(A) The ILC2 cluster from Figure 4.3B/C was subjected to further unsupervised clustering that divided ILC2s into two subsets (subset 1 and 2). Heatmap shows the top 20 genes that are differentially expressed by the subsets 1 and 2. **(B-C)** Heatmap shows all the genes that are differentially expressed ($p \leq 0.05$) by the adult ILC2 subsets. Genes that have higher expression in subset 1 than 2 **(B),** as well as genes that have higher expression in subset 2 than 1 **(C)** are shown. The genes selected and shown in the heatmap are found by the FindMarkers function with modification in the Seurat package. **(D)** Heatmap shows the expression of the ILC(2)-associated genes by the subsets 1 and 2. The arrows point to the genes that are mentioned in the text.

4.2.5 Adult Lung ILC2s Are Divided into IL-18Rα$^+$ST2$^-$ and IL-18Rα$^-$ST2$^+$ Subsets

The two ILC2 subsets detected by the scRNA-seq highly expressed *Thy1*, *Il7r* and *Yfp*, while differentially expressing *Il1rl1* and *Il18r1*. Flow cytometric analysis of Lin$^-$Tbet$^-$Thy1$^+$CD127$^+$RORγt$^-$ cells in adult Rorα-YFP mouse lungs revealed two subsets, namely the IL-18Rα$^-$ST2$^+$ conventional ILC2s and a small IL-18Rα$^+$ST2$^-$ subset (Figure 4.5A). About a half of the IL-18Rα$^+$ST2$^-$ cells were YFP$^+$. The IL-18Rα$^+$ST2$^-$YFP$^+$ cells were negative for CD25, KLRG1 and IL-25R and had low expression of GITR and GATA-3 compared to IL-18Rα$^-$ST2$^+$YFP$^+$ conventional ILC2s (Figure 4.5B). The IL-18Rα$^+$ST2$^-$YFP$^+$ cells increased in numbers along with conventional ILC2s following intranasal papain treatment (Figure 4.5A and D). To test the capacities of the IL-18Rα$^+$ST2$^-$YFP$^+$cells to produce cytokines, these cells and IL-18Rα$^-$ST2$^+$YFP$^+$ ILC2s were purified from naïve and papain-treated mouse lungs and stimulated with PMA and ionomycin. After 3 days, the culture supernatants were analyzed for multiple cytokines. The IL-18Rα$^+$ST2$^-$YFP$^+$ cells produced IL-5, albeit much lower amounts than conventional ILC2s (Figure 4.5D). The IL-18Rα$^+$ST2$^-$YFP$^+$ cells also produced small amounts of IL-2, IL-4, IL-17 and high amounts of MIP-1α and MIP-1β, whereas IFNγ and TNFα were undetectable.

The same two subsets were also found in *Rag1*$^{-/-}$ mice (Figure 4.6A). In agreement with the results obtained with Rorα-YFP mouse lung analysis, the IL-18Rα^{+}ST2^{-} cells from naïve *Rag1*$^{-/-}$ mice also produced small amounts of IL-5, IL-2, IL-4, IL-17, MIP-1α and MIP-1β compared to IL-18Rα^{-}ST2^{+} ILC2s (Figure 4.6B) and expanded after intranasal papain injections (Figure 4.6A and C).

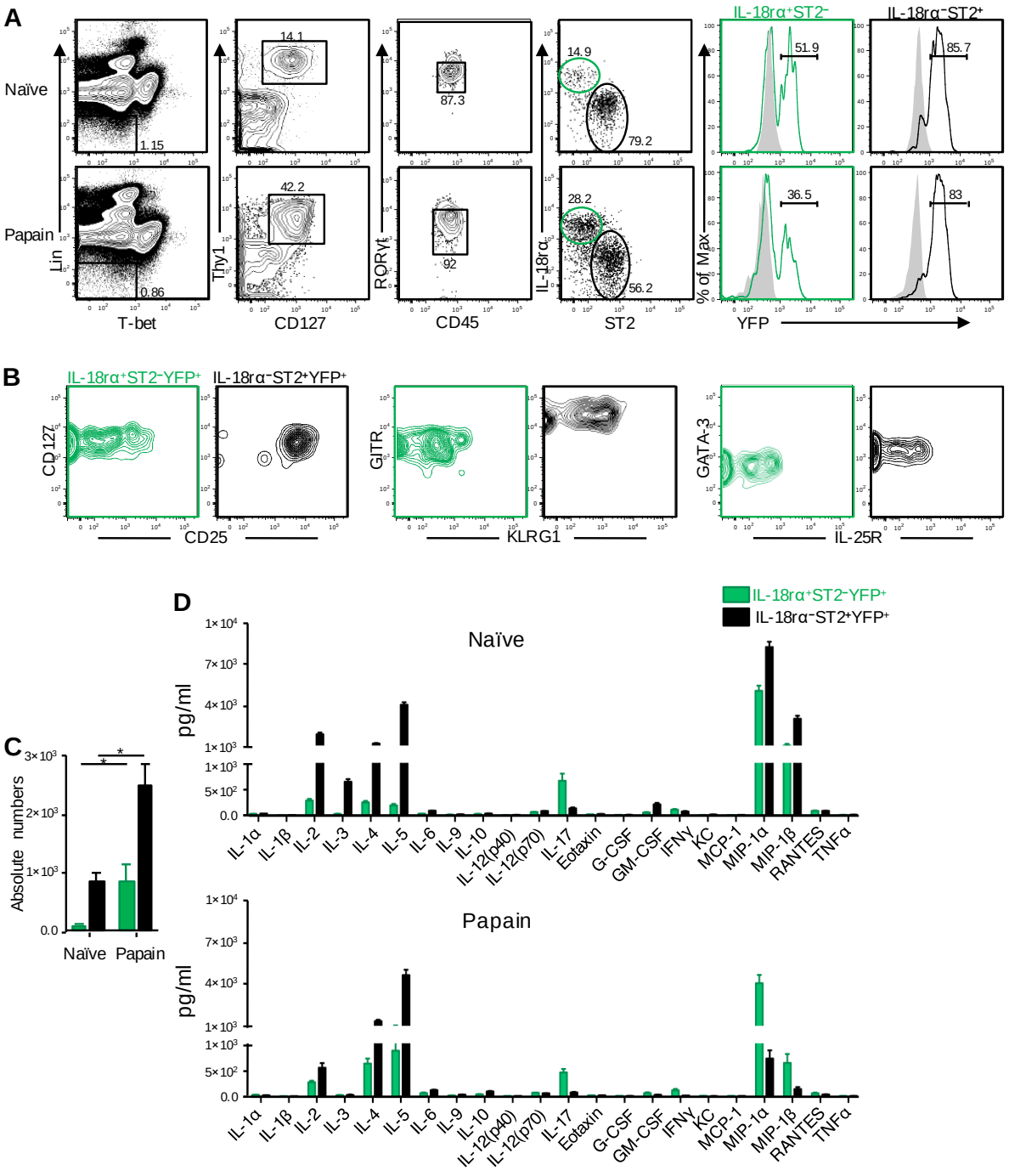

A
Naïve
Papain
Lin
T-bet
1.15
0.86
Thy1
CD127
14.1
42.2
RORγt
CD45
87.3
92
IL-18rα
ST2
14.9
79.2
28.2
56.2
IL-18rα+ST2-
IL-18rα-ST2+
% of Max
YFP
51.9
36.5
85.7
83
B
IL-18rα+ST2-YFP+
IL-18rα-ST2+YFP+
CD127
CD25
GITR
KLRG1
GATA-3
IL-25R
C
Absolute numbers
Naïve
Papain
D
IL-18rα+ST2-YFP+
IL-18rα-ST2+YFP+
Naïve
Papain
pg/ml
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12(p40)
IL-12(p70)
IL-17
Eotaxin
G-CSF
GM-CSF
IFNγ
KC
MCP-1
MIP-1α
MIP-1β
RANTES
TNFα

Figure 4.5 Flow cytometric and Functional Analyses Confirm Two Distinct Adult Lung ILC2 Subsets in RORα-YFP Mice.
(A) CD45$^+$Lin$^-$Tbet$^-$Thy1$^+$CD127$^+$RORγt$^-$ cells were sequentially gated and divided into IL-18Rα$^+$ST2$^-$ (green) and IL-18Rα$^-$ST2$^+$ (black) subsets and their expression of YFP in adult naïve (top) and papain-treated (bottom) lung RORα-YFP and B6 control (filled grey) mice is shown by histograms. **(B)** The expression of CD127 and CD25 (left), GITR and KLRG1 (middle) as well as GATA-3 and IL-25R (right) by the IL-18Rα$^+$ST2$^-$YFP$^+$ (green) and IL-18Rα$^-$ST2$^+$YFP$^+$ (black) in **A** (top) are shown by contour plots. **(C)** Absolute numbers of IL-18Rα$^+$ST2$^-$YFP$^+$ (green) and IL-18Rα$^-$ST2$^+$YFP$^+$ (black) in **A** in naïve and papain-treated lungs are shown in bar graphs. **(D)** CD45$^+$Lin$^-$Thy1$^+$CD127$^+$YFP$^+$ cells from adult naïve (top) and papain-treated (bottom) lung RORα-YFP mice were sorted into IL-18Rα$^+$ST2$^-$ (green) and IL-18Rα$^-$ST2$^+$ (black) cells and stimulated for 72 hours with PMA and ionomycin. The amounts of cytokines and chemokines in the culture supernatants were determined.

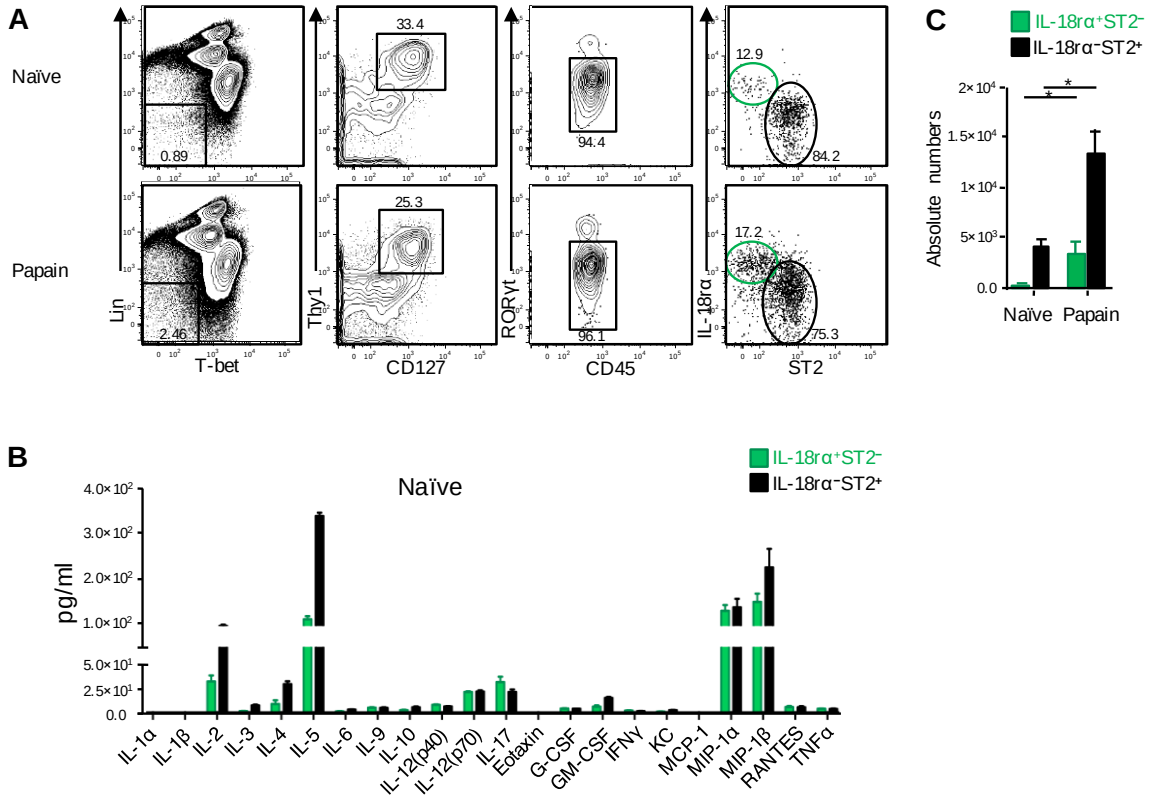


Figure 4.6 Flow Cytometric and Functional Analyses Confirm Two Distinct Adult Lung ILC2 Subsets in *Rag1*$^{-/-}$ Mice
(A) CD45$^+$Lin$^-$Tbet$^-$Thy1$^+$CD127$^+$RORγt$^-$ cells from adult naïve (top) and papain-treated (bottom) lung in *Rag1*$^{-/-}$ mice were sequentially gated and divided into IL-18Rα$^+$ST2$^-$ (green) and IL-18Rα$^-$ST2$^+$ (black) subsets. **(B)** CD45$^+$Lin$^-$Thy1$^+$CD127$^+$ cells from adult naïve lung *Rag1*$^{-/-}$ mice as in **A** (top) were sorted into IL-18Rα$^+$ST2$^-$ (green) and IL-18Rα$^-$ST2$^+$ (black) cells and stimulated for 72 hours with PMA and ionomycin. The amounts of cytokines and chemokines in the culture supernatants were determined. **(C)** Absolute numbers of IL-18Rα$^+$ST2$^-$ (green) and IL-18Rα$^-$ST2$^+$ (black) in **A** in naïve and papain-treated lungs are shown in bar graphs.

4.2.6 Adult Lung IL-18Rα^{+}ST2^{-} ILCs Express TCF-1

The IL-18Rα^{+}ST2^{-} cells did not express mature ILC2 markers and produced very little cytokines suggesting that they might be immature ILCs. In addition, the scRNA-seq gene expression profile of these cells indicated that a fraction of them express *Tcf7* (Figure 4.7A, C and D). Therefore, we analyzed their expression of *Tcf7*, using *Tcf7*^{EGFP} mice. Consistent with scRNA-seq results, about a half of the IL-18Rα^{+} cells were GFP^{+} (Figure 4.7A), whereas the conventional IL-18Rα^{-}ST2^{+} ILC2s were negative. The IL-18Rα^{+}ST2^{-}*Tcf7*$^{EGFP+}$ cells along with the conventional ILC2s expanded after intranasal papain injections. ILC3s express low levels of *Tcf7* in *Tcf7*^{EGFP} mice (Yang et al., 2015b). To exclude the rare ILC3s in our analysis and also examine TCF-1 protein expression by IL-18Rα^{+}ST2^{-} cells, we performed intracellular transcription factor staining of T-bet and RORγt and TCF-1 of adult lung cells (Figure 4.7B). We confirmed that IL-18Rα^{+}ST2^{-} cells express TCF-1, but not T-bet and RORγt, and expand after intranasal papain injections. Whereas, conventional IL-18Rα^{-}ST2^{+} ILC2s were negative for the expression of TCF-1.

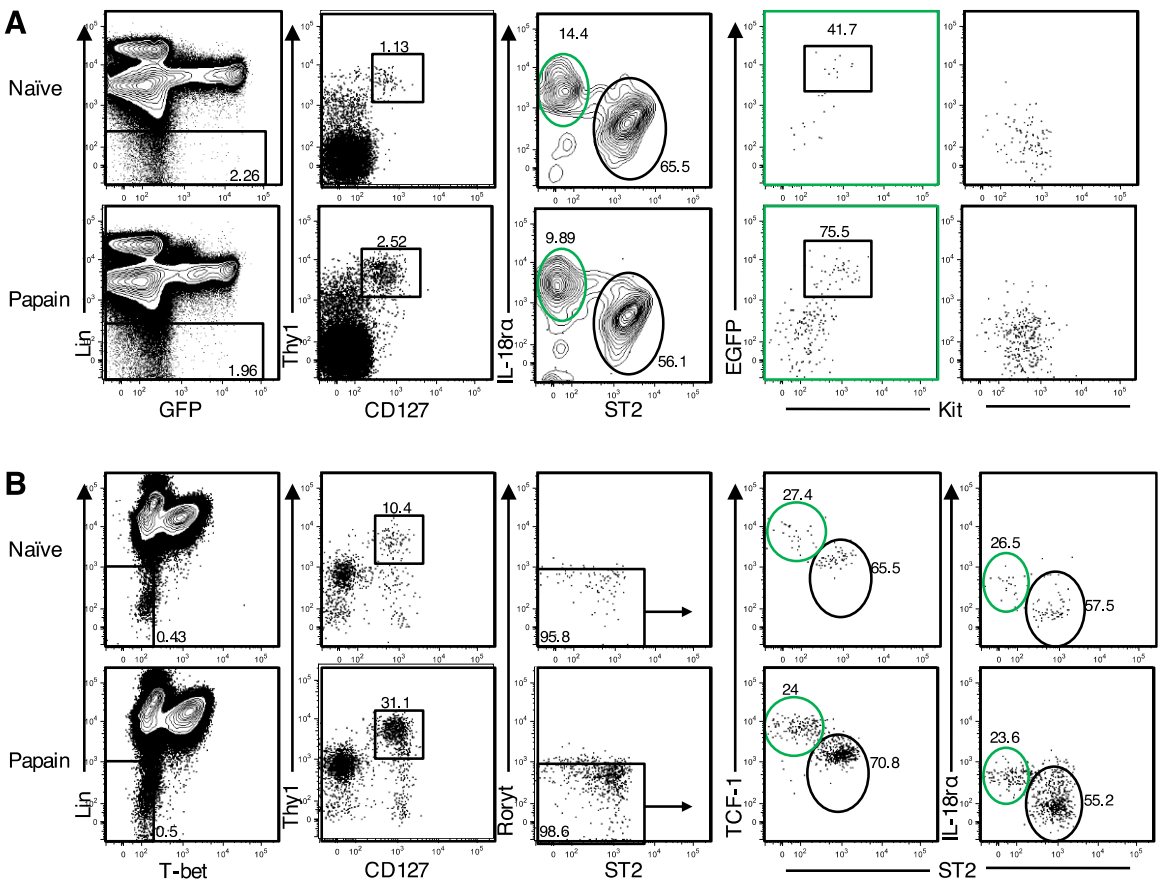


Figure 4.7 Flow Cytometric Analysis of Tcf7EGFP Mice Shows the Expression of EGFP by IL-18Rα+ST2− ILCs in Adult Lungs

(A) Adult lung Lin^{-}Thy1^{+}CD127^{+} cells from naïve (top) and papain-treated (bottom) *Tcf7*^{EGFP} mice were sequentially gated and divided into IL-18Rα^{+}ST2^{-} (green) and IL-18Rα^{-}ST2^{+} (black) subsets, and analyzed for the expression of EGFP and Kit. **(B)** Adult lung Lin^{-}Tbet^{-}Thy1^{+}CD127^{+}RORγt^{-} cells from naïve (top) and papain-treated (bottom) *Tcf7*^{EGFP} mouse lungs were sequentially gated and analyzed for the expression of TCF-1 and ST2 (left) as well as IL-18Rα and ST2 (right).

4.2.7 Adult Lung IL-18Rα^{+}ST2^{-} Are Not Dependent on CD127 Signaling for Their Development

To compare the developmental requirements of the IL-18Rα^{+}ST2^{-} versus IL-18Rα^{-}ST2^{+} ILC2s, we developed RORα-CD127cKO mice, by crossing *Rora-IRES-Cre* (Chou et al., 2013) and *Il7r*$^{fl/fl}$ mice, which have *loxP* sites flanking exon 3 of *Il7r*. In these RORα-CD127cKO mice, cells expressing *Rorα* during their development will have deletion of *Il7r* exon 3 resulting in their defective CD127 expression and signaling. Lung and intestinal ILC2 development were severely impaired in RORα-CD127cKO. This was to be expected as ILC2s strictly depend on CD127 signaling for their development and maintenance (Hoyler et al., 2012). In contrast, the B, T and Treg cell populations in the spleen and lung of RORα-CD127cKO were unaffected (Figure 4.8A). NK cells and ILC1s, which do not require CD127 signaling for their development (Geiger and Sun, 2016; Klose et al., 2014), also developed normally in these mice. The number of ILC3s (in the small intestine) in these mice were lower than controls but this did not reach statistical significance.

Flow cytometric analysis of Lin^{-}Tbet^{-}Thy1^{+}CD127^{+}RORγt^{-} cells in naïve and papain-treated adult RORα-CD127cKO mouse lungs showed that IL-18Rα^{+}ST2^{-} cells were not reduced compared to the same subset in the RORα-YFP mouse lungs (Figure 4.8B and C). Although, only about a half of the IL-18Rα^{+}ST2^{-} cells were YFP^{+} in the RORα-YFP mice (Figure 4.5A), the lack of any reduction in naïve and papain-treated RORα-CD127cKO mouse lungs, and considering the severe reductions of ILC2s in these mice, indicated that IL-18Rα^{+}ST2^{-} are less dependent on CD127 signaling for their development than ILC2s.

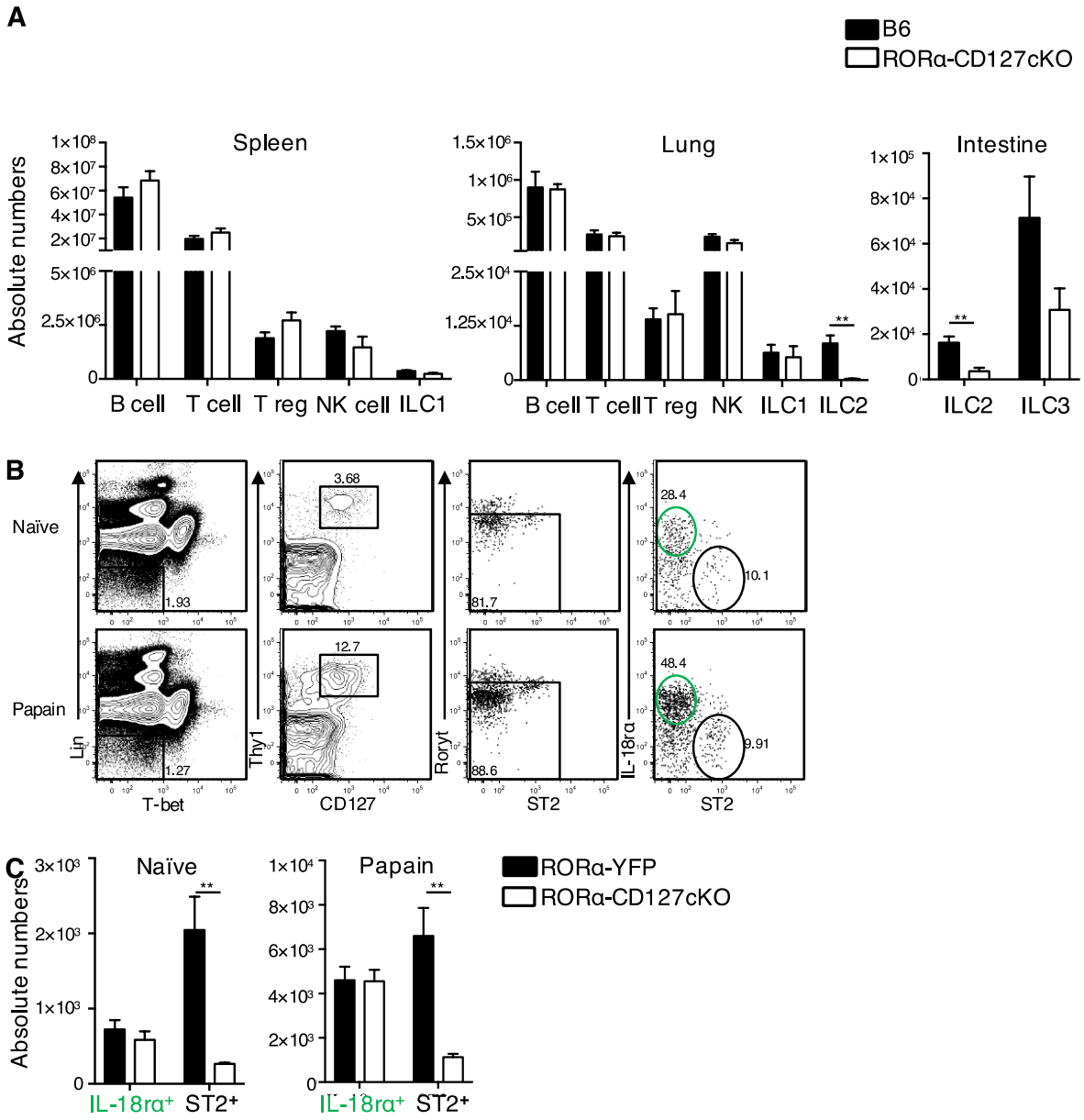


Figure 4.8 Flow Cytometric Analysis of RORα-CD127cKO Mice Shows that IL-18Rα+ST2− ILCs in Adult Lung Are Not Dependent on IL-7 Signaling for Their Development

(A) Absolute numbers of B, T, Treg, NK cells and ILC1s in the spleen (left), B, T, Treg, NK cells, ILC1s and ILC2s in the lung (middle), and ILC2s and ILC3s in the intestine (right) in B6 (black) and RORα-CD127cKO (white) mice are shown in bar graphs. (B) Adult lung Lin$^-$Tbet$^-$Thy1$^+$CD127$^+$RORγt$^-$ cells from naïve (top) and papain-treated (bottom) RORα-CD127cKO mice were sequentially gated and divided into IL-18Rα$^+$ST2$^-$ (green) and IL-18Rα$^-$ST2$^+$ (black) subsets. (C) Absolute numbers of IL-18Rα$^+$ST2$^-$ and IL-18Rα$^-$ST2$^+$ subsets (as gated in B) from naïve (left) and papain-treated (right) RORα-YFP (black) and RORα-CD127cKO (white) mice are shown in bar graphs.

4.3 Discussion

In this study, we generated a lineage tracer mouse model that marks ILCs with YFP, based on the expression of the transcription factor RORα. By scRNA-seq and flow cytometry we found $Lin^{-}CD127^{+}Thy1^{+}ST2^{-}IL\text{-}18R\alpha^{+}YFP^{+}$ cells in adult lungs that has an ILC progenitor-like phenotype. The adult $ST2^{-}IL\text{-}18R\alpha^{+}$ cells did not express T-bet and RORγt, the master transcription factors for ILC1s and ILC3s, respectively, and expressed low GATA-3 critical for ILC development (Hoyler et al., 2012; Zhu, 2017; Yagi et al., 2014; Serafini et al., 2014; Tindemans et al., 2014). The $ST2^{-}IL\text{-}18R\alpha^{+}$ population was also found in *Rag1*$^{-/-}$ mice confirming that it belongs to the ILC lineage. Unlike conventional ILC2s, $ST2^{-}IL\text{-}18R\alpha^{+}$ cells were $CD25^{-}GITR^{lo}$. They also lacked KLRG1 and IL-25R expression, excluding the possibility of them being inflammatory ILC2s (Huang et al., 2014, 2018a). These cells produced the same cytokines as ILC2s, albeit to much lower amounts, similar to the cytokine production capacities of BM ILC2Ps (Halim et al., 2012b). The $ST2^{-}IL\text{-}18R\alpha^{+}$ cells in our study are very similar to the unconventional ILC2s recently reported (Ricardo-Gonzalez et al., 2018). The lung $ST2^{-}IL\text{-}18R\alpha^{+}$*Il5*$^{+}$ cells in this report were considered functionally similar to skin ILC2s. It should be noted that the use of the RORα lineage tracer mice in our study, unlike the *Il5* reporter mice, enabled us to capture all $ST2^{-}IL\text{-}18R\alpha^{+}$ cells in the lung regardless of their basal activation status. In addition, the $ST2^{-}IL\text{-}18R\alpha^{+}$ cells in our study are similar to the systemic human ILC precursors and expressed the ILC progenitor associated transcription factor genes *Rora, Tcf7, Gata3, Id2* and *Runx3* as well as *Cd2, Cd7, Il7r, Il1r1* and *Il18r1* (Lim et al., 2017a). They both lacked any expression T-bet and RORγt and had low expression of GATA-3. The analysis of RORα-CD127cKO mice revealed that $ST2^{-}IL\text{-}18R\alpha^{+}$ cells were independent of CD127 signaling for their development. It is notable that BM ILC progenitors

are not dependent on IL-7 signaling for their development (Harly et al., 2017). Interestingly, ILC3s which are known to be dependent on CD127 signaling for their development and maintenance, were less severely reduced compared to ILC2s. This might be due to the IL-15 signaling compensating for the lack of IL-7 signaling (Robinette et al., 2015). Therefore, our results suggest that the $ST2^{-}$IL-$18R\alpha^{+}$ cells might be ILC progenitors. However, the ILC progenitor capacity of these cells should be confirmed using *in vivo* and *in vitro* differentiation assays.

Chapter 5: Single Cell Analysis of RORα-YFP Mice Revealed Heterogeneity of Neonatal Lung ILC2s

5.1 Introduction

ILC2s actively develop during the neonatal period (Ghaedi et al., 2016) and become long-lived tissue-resident cells (Gasteiger et al., 2015) in mucosal tissues including the lung. Neonatal lung ILC2s proliferate and become activated due to the spontaneous release of IL-33 in the lung (de Kleer et al., 2016; Saluzzo et al., 2017; Steer et al., 2017). Thereafter, ILC2s become long-lived tissue-resident cells (Gasteiger et al., 2015) in mucosal tissues including the lung. Therefore, the analysis of neonatal lung ILC2s not only provides insights into their heterogeneity after physiological activation with IL-33 but also their lung-specific developmental pathways during the most active phase of their generation. In order to study lung neonatal ILC2 complexity, we adopted the same strategy for our scRNA-seq analysis of RORα-YFP neonatal mouse lung as we did for adult mice. By this approach, we could further confirm that a distinct ILC subset resembling BM ILC progenitors is present in neonatal lung, which might actively contribute to the generation of ILC2s in the lung during this period. In addition, we could identify two alternative effector fates of ILC2 differentiation, namely pro-inflammatory and tissue-repairing ILC2s.

5.2 Results

5.2.1 RORα Lineage Tracing Marks Neonatal Innate Lymphocytes Including Lung ILC2s

In agreement with the efficient labeling of adult lung ILC2s by YFP, most (>80%) ILC2s, defined as $Lin^{-}GATA\text{-}3^{+}ST2^{+}Thy1^{+}$ (Figure 5.1A) or $Lin^{-}CD127^{+}Thy1^{+}ST2^{+}CD25^{+}$ (Figure 5.1B) were

also YFP^+ in neonatal (12-day-old) mouse lung. In addition, the Lin^-YFP^+ cells in neonatal lungs, were $ROR\gamma t^-$ and included GATA-$3^{hi}Thy1^+$, GATA-$3^{lo}Thy1^+$ and GATA-3^-Thy1^- cells (Figure 5.1C). The $Thy1^+$ cells included $ST2^+CD25^+$ ILC2s and a small fraction of $ST2^-CD25^-$ cells. The GATA-3^-Thy1^- cells were negative for the expression of all lymphoid-associated markers tested (data not shown). These also suggested that Lin^-YFP^+GATA-$3^{lo}Thy1^+ST2^-CD25^-$ cells might be unconventional ILC2s.

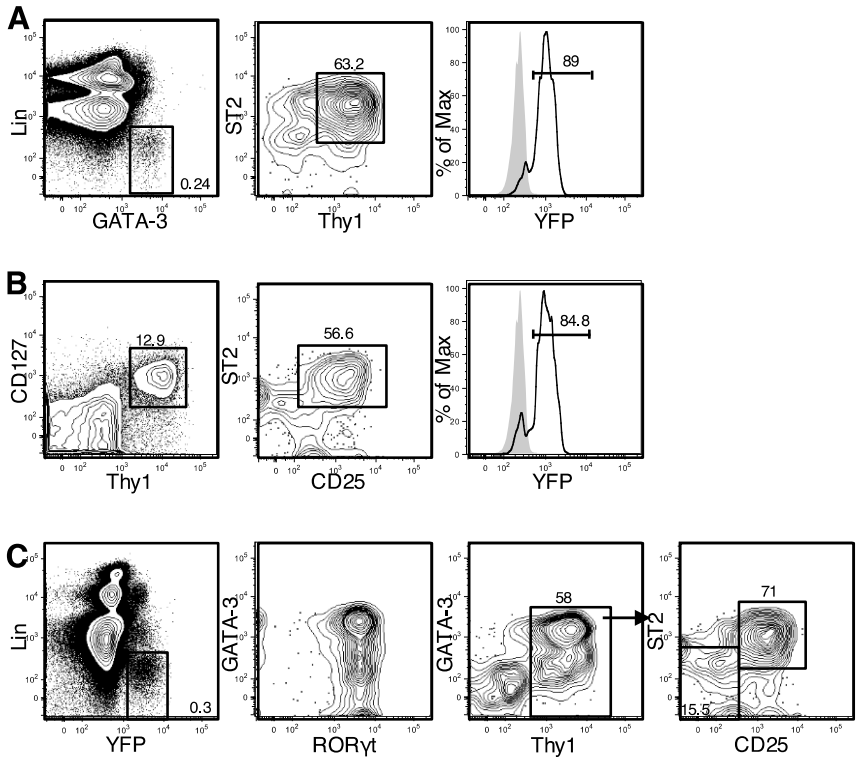
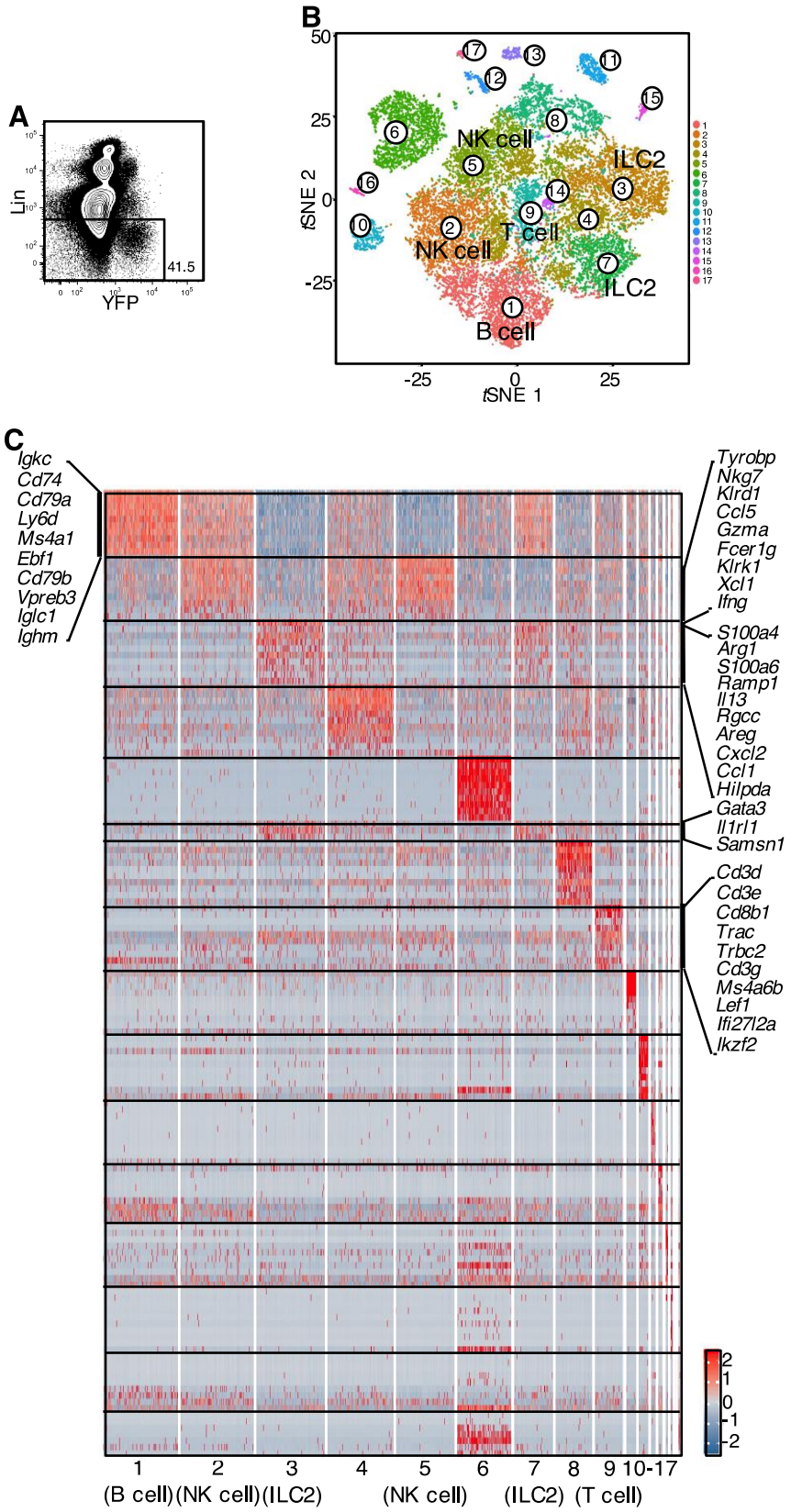


Figure 5.1 Flow Cytometric Analysis of RORα-YFP Mice Shows the Expression of YFP by Neonatal ILC2s

(A) Lung ILC2s from neonatal (12-day-old) mice were sequentially gated by $CD45^{lo/+}Lin^-$GATA-$3^+ST2^+Thy1^+$ in contour plots, and their expression of YFP in RORα-YFP (black line) and B6 control (filled grey) mice is shown by histograms. **(B)** Neonatal lung ILC2s were sequentially gated by $CD45^+Lin^-CD127^+Thy1^+ST2^+CD25^+$ in contour plots, and their expression of YFP in RORα-YFP (black line) and B6 control (filled grey) mice is shown by histograms. **(B)** The $CD45^{lo/+}Lin^-YFP^+$ cells from neonatal lungs were gated and analyzed for the expression of GATA-3 and RORγt as well as GATA-3 and Thy1. The lung $Lin^-YFP^+Thy1^+$ cells are further analyzed for the expression of ST2 and CD25. Data are representative of at least 3 independent experiments with at least 3 mice per group in each experiment.

5.2.2 ScRNA-seq Analysis Identifies ILC2s in Neonatal RORα-YFP Mouse Lungs

We analyzed all $CD45^{lo/+}Lin^{lo}$ lung cells (Figure 5.2A) from neonatal (12-day-old) RORα-YFP mice by scRNA-seq. Unsupervised clustering revealed 17 distinct clusters of stromal, myeloid, B, NK, T cell and ILC2 (Figure 5.2B and C and Table 5.1 Top 20 Differentially Expressed Genes by the 17 Cellular Clusters in). The ILC2 clusters were identified by their differential expression of ILC2-associated genes including *Arg1*, *Il13*, *Areg*, *Gata3* and *Il1rl1*. The ILC2 clusters were also evident on the *t*-SNE plot based on their high expression of *Rora*, *Gata3*, *Il7r*, *Il1rl1*, *Areg*, *Arg1*, *Il5* and *Il13* (Figure 5.2D). We combined the ILC2 clusters to carry out further analyses. We first confirmed that neonatal ILC2s are activated by analyzing differentially expressed genes by neonatal and naïve adult ILC2s. Consistent with the previous reports (de Kleer et al., 2016; Saluzzo et al., 2017; Steer et al., 2017), our analysis showed that neonatal ILC2s are expressing higher amounts of *Arg1*, *Il13*, *Il5* and *Klrg1* than adult ILC2s (Figure 5.3A).


A
Lin
YFP
41.5
B
tSNE 2
tSNE 1
NK cell
ILC2
T cell
NK cell
ILC2
B cell
C
Igkc
Cd74
Cd79a
Ly6d
Ms4a1
Ebf1
Cd79b
Vpreb3
Iglc1
Ighm
Tyrobp
Nkg7
Klrd1
Ccl5
Gzma
Fcer1g
Klrk1
Xcl1
Ifng
S100a4
Arg1
S100a6
Ramp1
Il13
Rgcc
Areg
Cxcl2
Ccl1
Hilpda
Gata3
Il1rl1
Samsn1
Cd3d
Cd3e
Cd8b1
Trac
Trbc2
Cd3g
Ms4a6b
Lef1
Ifi27l2a
Ikzf2
1 2 3 4 5 6 7 8 9 10-17
(B cell) (NK cell) (ILC2) (NK cell) (ILC2) (T cell)

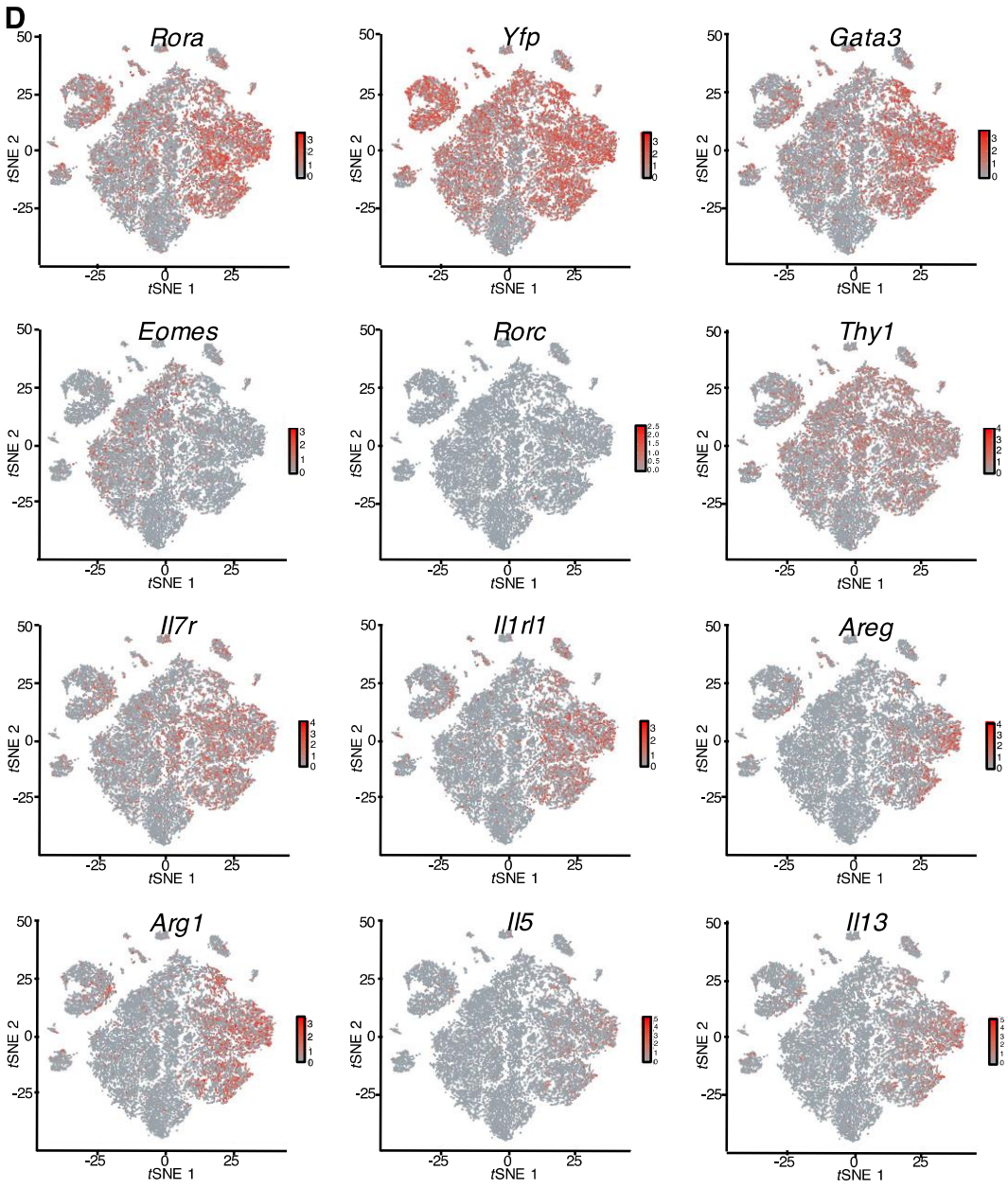


Figure 5.2 ScRNA-seq Analysis Identifies ILC2s in Neonatal Mouse Lungs

(A) Gating strategy for purification of Lin^{lo} cells from 12-day-old neonatal RORα-YFP mouse lungs for scRNA-seq analysis. **(B)** *t*-Distributed stochastic neighbor embedding (*t*-SNE) plot shows distinct clusters within the 21,256 sequenced cells. **(C)** Heatmap of the differentially expressed genes by the clusters from **B**. Top 10 differentially expressed transcripts of the lymphoid lineages are shown. **(D)** *t*-SNE plots showing expression of the indicated individual genes.

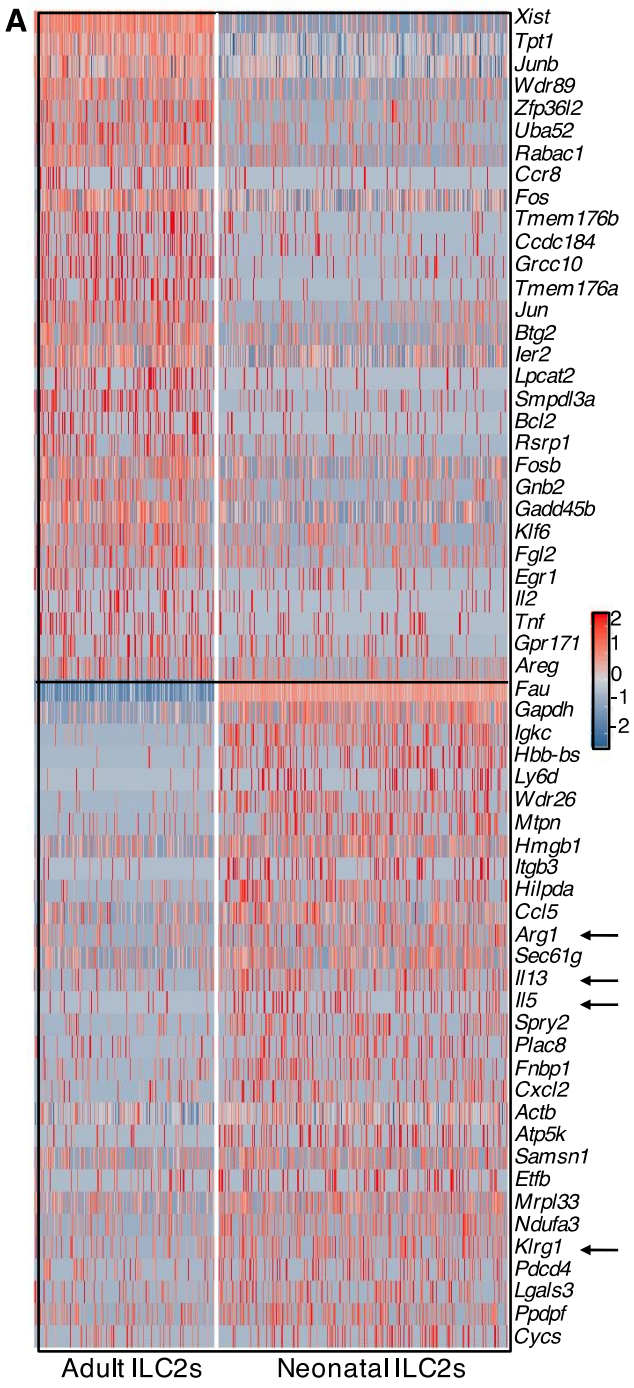


Figure 5.3 Differential Gene Expression Analysis of the Adult and Neonatal Lung ILC2s Subsets

(A) Heatmap shows the top genes that are differentially expressed ($p \leq 0.05$) by the adult (combined adult ILC2s from Figure 4.4A) and neonatal ILC2s (combined ILC2 subsets from Figure 5.4A). The arrows point to the activation-associated genes *Arg1*, *Il5*, *Il13* and *Klrg1*.

Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Cluster 8	Cluster 9	Cluster 10	Cluster 11	Cluster 12	Cluster 13	Cluster 14	Cluster 15	Cluster 16	Cluster 17
Igkc	*Tyrobp*	*S100a4*	*Spry2*	*Gzma*	*Eln*	*S100a4*	*2810417H13Rik*	*Cd3d*	*Hbb-bs*	*Ifitm1*	*Sftpa1*	*Bst2*	*Car1*	*Cldn5*	*Fscn1*	*Dcn*
Cd74	*Nkg7*	*Arg1*	*Pim1*	*Klrd1*	*Aspn*	*Arg1*	*Hmgb2*	*Cd3e*	*Hba-a1*	*Ccl6*	*Lyz2*	*Cox6a2*	*Vamp5*	*Egfl7*	*Zmynd15*	*Igfbp6*
Cd79a	*Klrd1*	*S100a6*	*Actb*	*Nkg7*	*Sparc*	*Rgcc*	*Stmn1*	*Cd8b1*	*Hba-a2*	*Ccl3*	*Sftpb*	*Siglech*	*Gm15915*	*Ramp2*	*Il12b*	*Upk3b*
Ly6d	*Ccl5*	*Ramp1*	*Kdm6b*	*Tyrobp*	*Bgn*	*Samsn1*	*Tuba1b*	*Trac*	*Hbb-bt*	*Ccl9*	*S100g*	*Smim5*	*Clec4d*	*Gng11*	*Cxcl16*	*Aebp1*
Ms4a1	*Igkc*	*Il13*	*Notch2*	*Fcer1g*	*Col3a1*	*Ltb4r1*	*Birc5*	*Trbc2*	*Alas2*	*Alox5ap*	*Wfdc2*	*Ccr9*	*Gstm5*	*Tmem100*	*Cacnb3*	*Gpc3*
Ebf1	*Gzma*	*Gata3*	*Abhd2*	*AW112010*	*Mustn1*	*Gata3*	*Ube2c*	*Cd3g*	*Snca*	*Mcpt8*	*Ager*	*Lair1*	*Aqp1*	*Sdpr*	*Basp1*	*Nkain4*
Cd79b	*Fcer1g*	*Samsn1*	*Pdcd4*	*Ccl5*	*Cyr61*	*S100a6*	*Tubb5*	*Ms4a6b*	*Ube2l6*	*Hdc*	*Sftpd*	*Plaur*	*Tspo2*	*Ecscr*	*Tm4sf5*	*Mgp*
Vpreb3	*Ly6d*	*Rgcc*	*Cbx3*	*Klrk1*	*Tgfbi*	*Cxcl2*	*Cks1b*	*Lef1*	*Bpgm*	*Il6*	*Cxcl15*	*Runx2*	*Gata2*	*Tm4sf1*	*Marcks*	*Rarres2*
Iglc1	*AW112010*	*Ltb4r1*	*Wtap*	*Cd7*	*Serpine2*	*Il1rl1*	*Top2a*	*Ifi27l2a*	*Fam46c*	*Lilr4b*	*Cldn18*	*Tcf4*	*Apoe*	*Scn7a*	*Cd83*	*Meg3*
Ighm	*Klrk1*	*Rgs1*	*Insig1*	*Klre1*	*Tagln*	*Ramp1*	*Hmgn2*	*Ms4a4b*	*Mkrn1*	*Osm*	*Cbr2*	*Alox5ap*	*Cox6b2*	*S100a16*	*Ccr7*	*Apoe*
Iglc3	*Ebf1*	*Klrg1*	*Slc39a1*	*Ctsw*	*Col1a2*	*Rora*	*Rrm2*	*Gm8369*	*Slc25a37*	*Cyp11a1*	*Krt18*	*Grn*	*Cdk6*	*Cdh5*	*Net1*	*Col1a1*
Iglc2	*Xcl1*	*Stab2*	*B4galt1*	*Il18r1*	*Col1a1*	*Areg*	*H2afx*	*Tcf7*	*Gpx1*	*Csf2rb*	*Sfta2*	*Ly6c2*	*Khk*	*Hpgd*	*Cd63*	*Sparc*
H2-Ab1	*Serpinb6b*	*Areg*	*Kmt2d*	*Ms4a4b*	*Fstl1*	*Gadd45b*	*H2afz*	*Ikzf2*	*Bnip3l*	*Csrp3*	*Chil1*	*Rnase6*	*Myb*	*Stmn2*	*Traf1*	*Igfbp5*
Cd24a	*Ctsw*	*Rora*	*Zc3hav1*	*Xcl1*	*Serpinh1*	*Klrg1*	*Cenpa*	*Lck*		*Lilrb4a*	*Cldn3*	*Plac8*	*Ifitm2*	*Cav1*	*Gclc*	*Col3a1*
Cd72	*Klre1*	*Cxcl2*	*Pan3*	*Serpinb6b*	*Pam*	*Nfkb1*	*Cdca3*	*Trbc1*		*Cd200r3*	*Anxa3*	*Irf8*	*Mt1*	*Calcrl*	*Epsti1*	*Serpinh1*
Spib	*Iglc2*	*Furin*	*Alkbh5*	*Sh2d1a*	*Fos*	*Hilpda*	*Ccnb2*	*Lat*		*Ier3*	*Lmo7*	*Pld4*	*Atpif1*	*Cd36*	*Cst3*	*Col1a2*
Siglecg	*Ifng*	*Ccl1*	*Supt16*	*Gimap4*	*Mfap4*	*Klk8*	*Smc2*	*Gimap3*		*Ccl4*	*Bex4*	*Ctsh*	*Gpx1*	*Crip2*	*Npc2*	*Ifitm3*
H2-Eb1	*Cd7*	*Ctla2a*	*Rpgrip1*	*Ncr1*	*Mfap2*	*Fam110a*	*Cenpf*	*Gimap4*		*Cd63*	*Emp2*	*Ctsb*	*Stmn1*	*Ifitm3*	*Klf6*	*Csrp2*
H2-Aa	*Gimap4*	*Hilpda*	*Ppp1r16b*	*Car2*	*Id3*	*Rgs1*	*Mki67*	*Rgs10*		*Ifitm2*	*Sftpc*	*Tyrobp*	*Plac8*	*Ifitm2*	*Serpinb6b*	*Fabp5*
Mzb1	*Ccl4*	*Gadd45b*	*Furin*	*Ifng*	*Thbs1*	*Il13*	*Dut*	*Bcl2*		*Apoe*	*Npc2*	*Psap*	*Nop10*	*Sepp1*	*Ccl5*	*Gsn*

Table 5.1 Top 20 Differentially Expressed Genes by the 17 Cellular Clusters in Figure 5.2 B and C

The table shows the top 20 genes that are highly expressed ($p \leq 0.01$) by each cluster in Figure 5.2B and C. The genes were selected by the FindMarkers function in the Seurat package.

5.2.3 Identification of Two Distinct ILC2 Subsets in Neonatal RORα-YFP Mouse Lungs by scRNA-seq Analysis

Further unsupervised clustering of the combined neonatal ILC2s, divided them into two subsets (1 and 2), each expressing a distinct set of genes (Figure 5.4A). Subset 1 mainly included ILC2s with high expression of *Il5*, *Il13*, *Arg1* and *Klrg1*, whereas subset 2 was highly enriched in *Areg* and *Icos* expressing ILC2s. A complete analysis of the transcripts that were differentially expressed between these two subsets indicated that the *Il5/Il13* expressing subset also had slightly higher expression of the activation marker *Cd69* (Figure 5.4B). Whereas, the *Areg/Icos* expressing subset had slightly higher expression of *Zbtb16, Kit* and *Il7r* (Figure 5.4A, C and D). *Zbtb16* encoding the transcription factor PLZF, is known to induce the upregulation of ICOS (Gleimer et al., 2012). The two subsets equally expressed *Id2*, *Gata3*, *Thy1* and *Il1rl1* (Figure 5.4D).

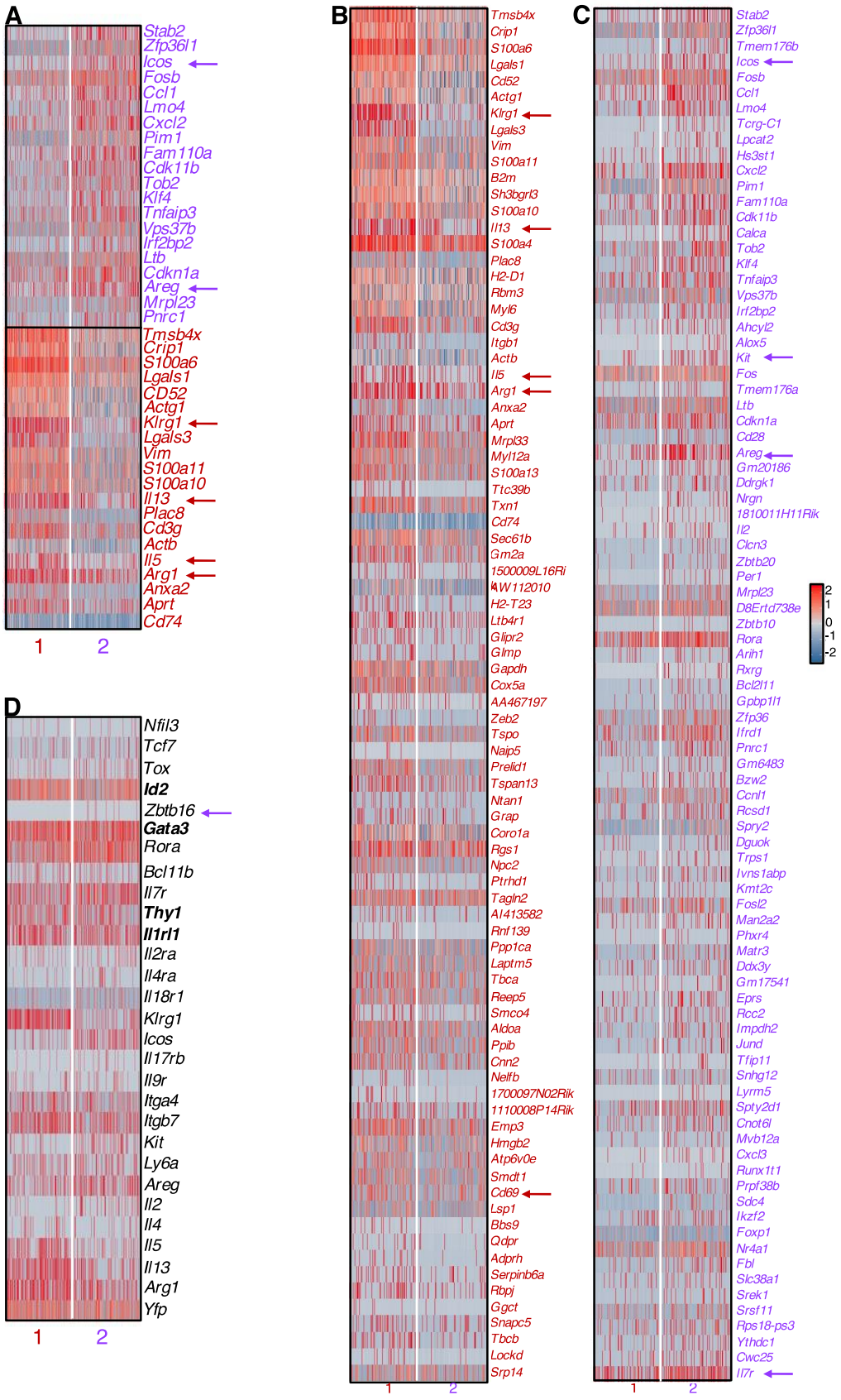

A
Stab2
Zfp36l1
Icos
Fosb
Ccl1
Lmo4
Cxcl2
Pim1
Fam110a
Cdk11b
Tob2
Klf4
Tnfaip3
Vps37b
Irf2bp2
Ltb
Cdkn1a
Areg
Mrpl23
Pnrc1
Tmsb4x
Crip1
S100a6
Lgals1
CD52
Actg1
Klrg1
Lgals3
Vim
S100a11
S100a10
Il13
Plac8
Cd3g
Actb
Il5
Arg1
Anxa2
Aprt
Cd74
1
2
B
Tmsb4x
Crip1
S100a6
Lgals1
Cd52
Actg1
Klrg1
Lgals3
Vim
S100a11
B2m
Sh3bgrl3
S100a10
Il13
S100a4
Plac8
H2-D1
Rbm3
Myl6
Cd3g
Itgb1
Actb
Il5
Arg1
Anxa2
Aprt
Mrpl33
Myl12a
S100a13
Ttc39b
Txn1
Cd74
Sec61b
Gm2a
1500009L16Ri
4W112010
H2-T23
Ltb4r1
Glipr2
Glmp
Gapdh
Cox5a
AA467197
Zeb2
Tspo
Naip5
Prelid1
Tspan13
Ntan1
Grap
Coro1a
Rgs1
Npc2
Ptrhd1
Tagln2
AI413582
Rnf139
Ppp1ca
Laptm5
Tbca
Reep5
Smco4
Aldoa
Ppib
Cnn2
Nelfb
1700097N02Rik
1110008P14Rik
Emp3
Hmgb2
Atp6v0e
Smdt1
Cd69
Lsp1
Bbs9
Qdpr
Adprh
Serpinb6a
Rbpj
Ggct
Snapc5
Tbcb
Lockd
Srp14
1
2
C
Stab2
Zfp36l1
Tmem176b
Icos
Fosb
Ccl1
Lmo4
Tcrg-C1
Lpcat2
Hs3st1
Cxcl2
Pim1
Fam110a
Cdk11b
Calca
Tob2
Klf4
Tnfaip3
Vps37b
Irf2bp2
Ahcyl2
Alox5
Kit
Fos
Tmem176a
Ltb
Cdkn1a
Cd28
Areg
Gm20186
Ddrgk1
Nrgn
1810011H11Rik
Il2
Clcn3
Zbtb20
Per1
Mrpl23
D8Ertd738e
Zbtb10
Rora
Arih1
Rxrg
Bcl2l11
Gpbp1l1
Zfp36
Ifrd1
Pnrc1
Gm6483
Bzw2
Ccnl1
Rcsd1
Spry2
Dguok
Trps1
Ivns1abp
Kmt2c
Fosl2
Man2a2
Phxr4
Matr3
Ddx3y
Gm17541
Eprs
Rcc2
Impdh2
Jund
Tfip11
Snhg12
Lyrm5
Spty2d1
Cnot6l
Mvb12a
Cxcl3
Runx1t1
Prpf38b
Sdc4
Ikzf2
Foxp1
Nr4a1
Fbl
Slc38a1
Srek1
Srsf11
Rps18-ps3
Ythdc1
Cwc25
Il7r
1
2
2
1
0
-1
-2
D
Nfil3
Tcf7
Tox
Id2
Zbtb16
Gata3
Rora
Bcl11b
Il7r
Thy1
Il1rl1
Il2ra
Il4ra
Il18r1
Klrg1
Icos
Il17rb
Il9r
Itga4
Itgb7
Kit
Ly6a
Areg
Il2
Il4
Il5
Il13
Arg1
Yfp
1
2

Figure 5.4 ScRNA-seq Analysis Identifies Two Distinct ILC2 Subsets in Neonatal Mouse

(A) The ILC2 clusters from Figure 5.2B/C were combined and subjected to further unsupervised clustering, which divided them into two subsets (subset 1 and 2). Heatmap shows top 20 genes that are differentially expressed by the subsets 1 and 2. (B-C) Heatmap shows all the genes that are differentially expressed ($p \leq 0.05$) by the neonatal ILC2 subsets. Genes that have higher expression in subset 1 than 2 **(B),** as well as genes that have higher expression in subset 2 than 1 **(C)** are shown. The genes selected and shown in the heatmap are found by the FindMarkers function with modification in the Seurat package. **(D)** Heatmap shows the expression of the ILC(2)-associated genes by the subsets 1 and 2. The arrows point to the genes that are mentioned in the text.

5.2.4 Trajectory Analysis Revealed ILC Progenitor-Like Cells in Neonatal Lungs

Lung ILC2 development starts soon after birth (Ghaedi et al., 2016). To examine the developmental relationship between the two neonatal ILC2 subsets above, individual neonatal ILC2s were positioned along a differentiation trajectory according to the virtual progression of their transcriptome (Figure 5.5A) (Trapnell et al., 2014; Trapnell, 2015). This analysis placed individual ILC2s into 5 different states. State 1 was enriched for cells expressing ILC progenitor-associated genes including *Il7r, Kit, Tcf7* (Yang et al., 2015b; Obaldia and Bhandoola, 2015), *Tox* (Seehus et al., 2015b) and *Zbtb16* (Constantinides et al., 2014) and lacked expression of the mature ILC2-associated genes including *Il1rl1*, *Areg*, *Il5*, *Il13*, *Arg1* and *Klrg1* (Figure 5.5A and B), suggesting that these cells are ILC progenitors residing in neonatal lungs. Analyses of the genes differentially expressed along cell fate progression from state 1 to states 4 and 5 revealed progressive downregulation of the ILC-progenitor-associated genes *Tcf7* (Yang et al., 2015b), *Tox* (Seehus et al., 2015a) and *Runx3* (Ebihara et al., 2015) as well as *Il18r1* (Figure 5.5C). Mature ILC2-associated genes *Il1rl1*, *Areg*, *Arg1*, *Il5*, *Il13* and *Klrg1* showed progressive up-regulation along their cell fate progression. The state 1 progenitor cells appeared to differentiate into two distinct ILC2 effector subsets (states 4 and 5) (Figure 5.5A and B). State 4 was enriched for cells expressing *Il5, Il13, Arg1* and *Klrg1*, whereas state 5 was enriched for cells expressing *Areg* and *Icos*. State 2 seemed to be a transitional state connecting progenitors to the mature cells in states 4 and 5. State 3 did not

have any distinctive features from states 4 and 5 (data not shown). These results suggested that an ILC progenitor population exists in the neonatal lung that gradually differentiates into two alternative effector fates, namely pro-inflammatory and tissue-repairing ILC2s.

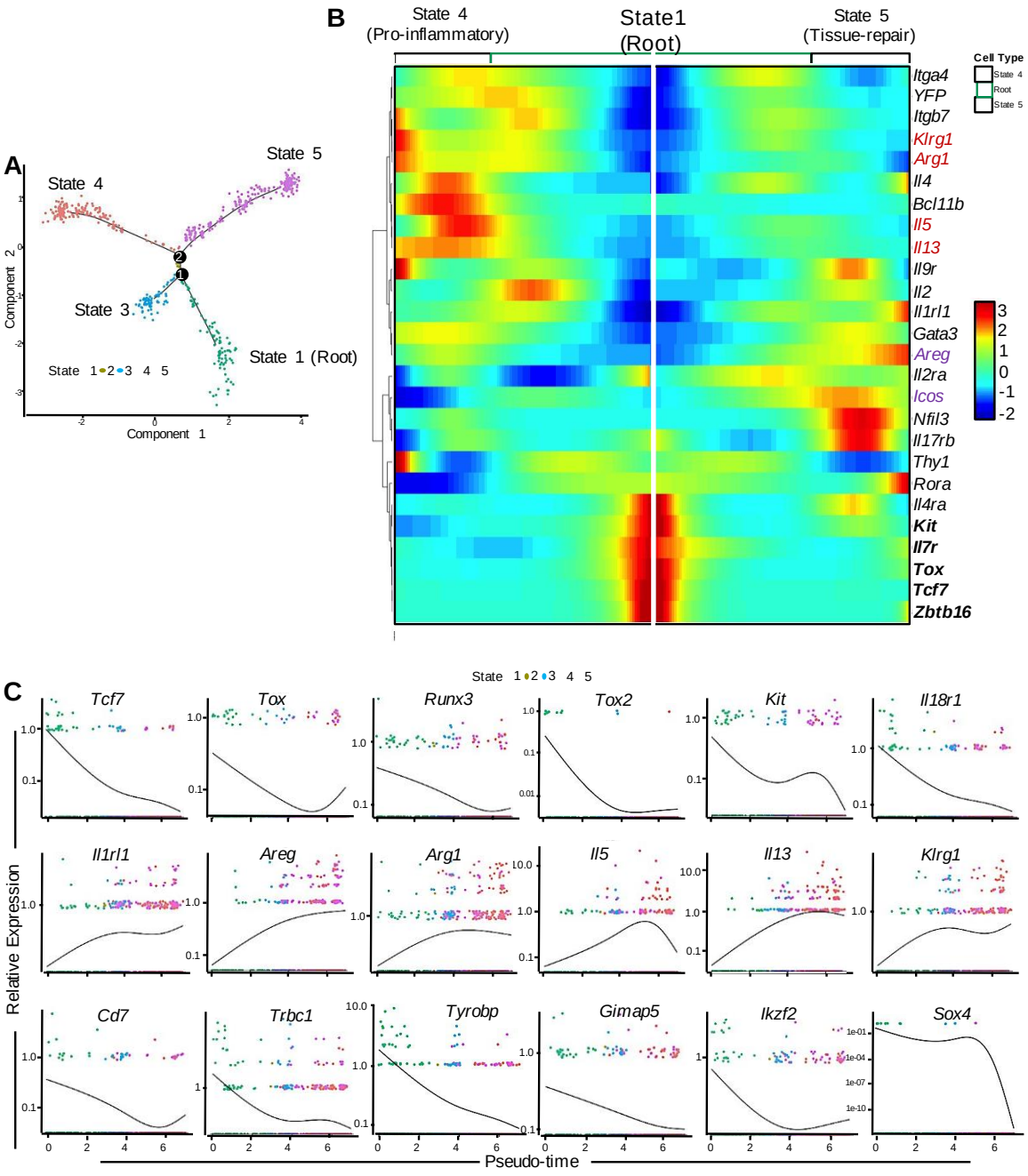


Figure 5.5 Trajectory Analysis of ScRNA-seq Neonatal Lung ILC2s Identifies Effector ILC2 Subsets and ILC Progenitor-Like Cells in the Neonatal Lung

(A) Single cell trajectory was constructed from the neonatal ILC2 dataset in Figure 5.4A using the Monocle package and neonatal ILC2s were ordered in pseudotime along a tree-like differentiation trajectory. Five distinct segments or states of the trajectory were identified. **(B)** Heatmap of the expression of ILC2-associated genes in the indicated states in **A**. **(C)** Changes in the expression of the indicated ILC2-associaetd genes along cell fate progression in pseudotime, from state 1 to states 4 and 5 in **A**.

5.2.5 Distinct Effector ILC2 Subsets and ILC Progenitor-Like cells in Neonatal Mouse Lungs

The scRNA-seq analyses above showed two subsets of effector ILC2s and a population of ILC progenitor-like cells in neonatal lungs. To further study the ILC progenitor-like cells, we carried out flow cytometric analyses of neonatal (12-day-old) lungs and adult BM as well as neonatal BM of *Tcf7*^{EGFP} mice (Figure 5.6A). The Lin$^-$Thy1$^+$CD127$^+$ cells were divided into two subsets based on the expression of ST2. The lung ST2$^-$ cells highly expressed EGFP and included IL-18Rα$^+$Kit$^+$ and IL-18Rα$^-$Kit$^-$ cells. The ST2$^+$ ILC2s were EGFP negative and mostly IL-18Rα negative. In the adult BM, ILC progenitors defined as Lin$^-$Thy1$^+$CD127$^+$EGFP$^+$ST2$^-$ were mostly IL-18Rα and Kit positive. In contrast, the Lin$^-$Thy1$^+$CD127$^+$ST2$^+$ ILC2Ps were EGFP$^-$IL-18Rα$^-$Kit$^-$. In the neonatal BM, only very small numbers of Lin$^-$Thy1$^+$CD127$^+$ were found and included similar cell populations as neonatal lung and adult BM. These results showed that early ILC progenitor-like cells in the lung are similar to early ILC progenitors, but not ILC2Ps in the BM.

Consistent with the scRNA-seq data, neonatal B6 lung ILC2s (Lin$^-$ST2$^+$Thy1$^+$CD127$^+$) were divided into two subsets based on the expression of KLRG1 and ICOS (Figure 5.6B). The KLRG1$^-$ICOS$^+$ had slightly higher expression of CD127. Unlike neonatal ILC2s, the divergence between KLRG1 and ICOS was not clear in adult lung ILC2s after papain treatments. (Figure 5.6C). We purified the neonatal KLRG1$^+$ICOS$^-$ and KLRG1$^-$ICOS$^+$ subsets as well as KLRG1$^+$ICOS$^+$ and KLRG1$^-$ICOS$^-$ cells and stimulated *in vitro* by a combination of IL-33 and IL-7 (Figure 5.6D). After the indicated time points, the KLRG1$^+$ICOS$^-$ produced much higher amounts of IL-5 and IL-13, compared to other subsets and minimally produced amphiregulin. The KLRG1$^-$ICOS$^+$ subset produced the highest amount of amphiregulin and did not produce much IL-5 and IL-13. These findings indicated that KLRG1 and ICOS expression distinguish two functionally distinct effector

subsets of neonatal ILC2s, namely IL-5/IL-13 cytokine-producing ILC2s and amphiregulin producing ones.

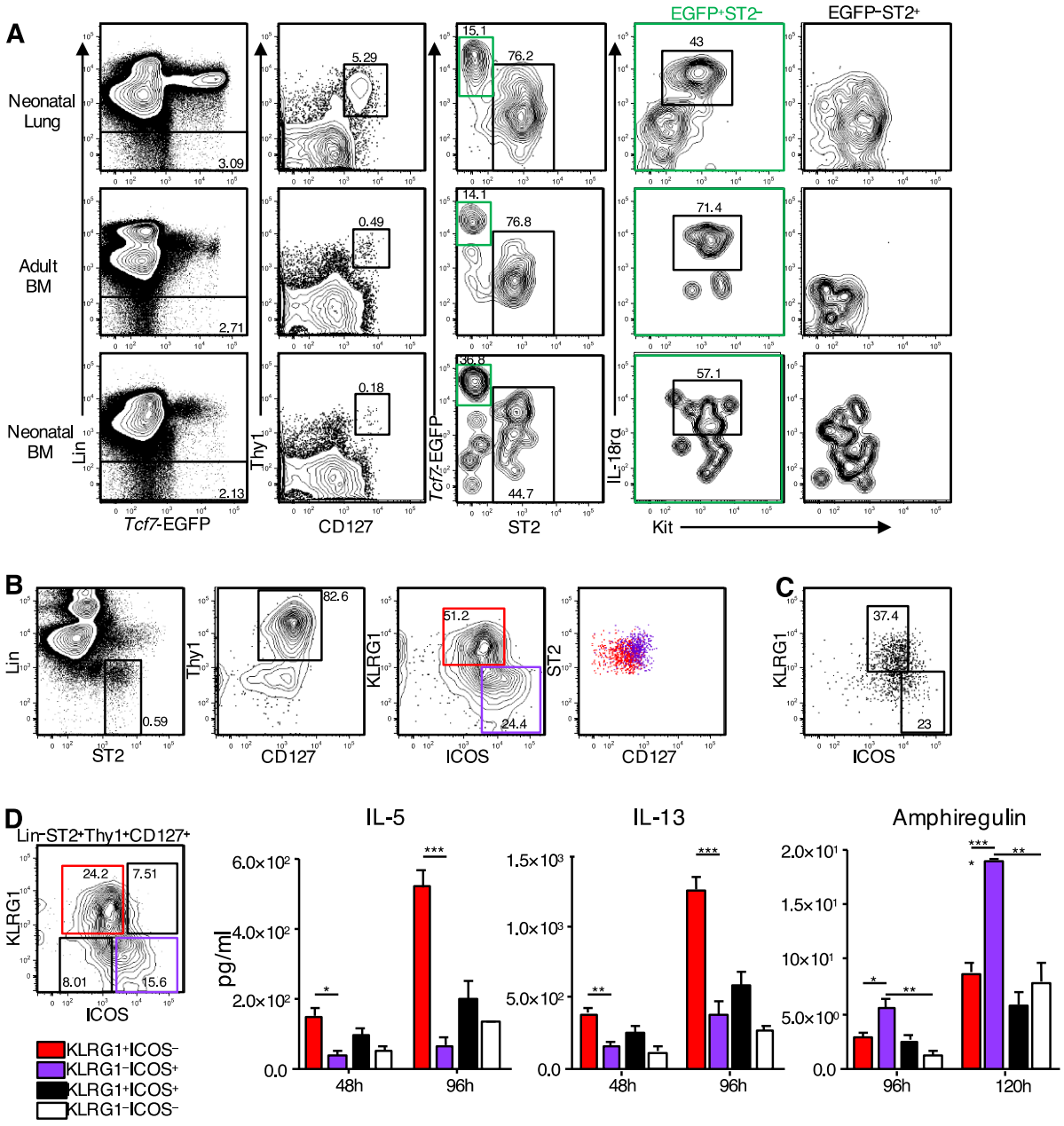


Figure 5.6 Flow Cytometric and Functional Analysis Shows ILC Progenitor-Like Cells and Distinct Effector ILC2 Subsets in Neonatal Mouse Lungs

(A) $CD45^+Lin^-Thy1^+CD127^+$cells from neonatal (12-day-old) lung, adult and neonatal (12-day-old) BM from *Tcf7*^{EGFP} mice were sequentially gated and divided into $EGFP^+ST2^-$ (green) and $EGFP^-ST2^+$ (black) subsets, and analyzed for the expression of IL-18Rα and Kit. **(B)** $CD45^+Lin^-ST2^+Thy1^+CD127^+$ cells from 12-day-old B6 mouse lungs were analyzed for KLRG1 and ICOS expression. The expression of ST2 and CD127 by the $KLRG1^+ICOS^-$ (red gate) and $KLRG1^-ICOS^+$ (purple) subsets were overlapped in dot plot. **(C)** $CD45^+Lin^-ST2^+Thy1^+CD127^+$ cells from papain treated adult B6 mouse lungs were analyzed for KLRG1 and ICOS expression. **(D)** $Lin^-ST2^+Thy1^+CD127^+$ cells from neonatal B6 lungs were divided into four subsets based on KLRG1 and ICOS expression, as shown in contour plot. Each subset was sorted and cultured for the indicated hours (hr) with IL-33 and IL-7 (1 ng/ml each for cytokine and 10 ng/ml for amphiregulin analysis). The amounts of IL-5, IL-13 and amphiregulin in the culture supernatants were determined by ELISA. Data in A are representative of 2 independent experiments. Data in B, C and D are representative of at least 3 independent experiments with 12 mice per group in each experiment. Mean ± SEM. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) were considered significant (two-tailed Student's t test).

5.3 Discussion

In this chapter, our scRNA-seq and flow cytometry analyses of neonatal RORα-YFP mice revealed three transcriptionally distinct ILC2 subsets; *Tcf7* expressing ILC progenitor-like, *Klrg1*/*Il5*/*Il13* and *Icos*/*Areg* expressing effector ILC2 subsets. We confirmed that the neonatal lung ILC progenitor-like cells are phenotypically similar to BM ILCPs as they are $Lin^{-}Thy1^{+}CD127^{+}Tcf7^{GFP+}ST2^{-}$. Both neonatal lung and adult BM ILCPs were also IL-18Rα^{+}Kit^{+}. ILC progenitor-like cells were more frequent in neonatal lungs than BM, suggesting that neonatal lung tissue might provide an important niche for the development of lung ILC2s. It remains to be determined whether these ILC progenitor-like cells are long lived resident cells and contribute to the ILC development in the lung of neonates and adult. It has been reported that hematopoietic stem cells (HSCs), common lymphoid progenitors (CLPs) and early thymic progenitors (ETPs) express IL-18Rα (Gandhapudi et al., 2015). IL-18 plus IL-7 promotes the expansion of HSCs and CLPs, and IL-18 alone can promote differentiation of ETPs into double negative stage 3 *in vitro*. In addition, IL-18Rα is one of the cytokine receptors upregulated during the transition of all-lymphoid progenitors to EILPs and ILCPs (Harly et al., 2017).

We divided the neonatal ILC2s into two functionally distinct subsets based on the expression of KLRG1 and ICOS. The KLRG1^{+} subset produced large amounts of IL-5 and IL-13, whereas the ICOS^{+} subset produced high amounts of amphiregulin. Considering that neonatal ILC2s are activated (de Kleer et al., 2016; Steer et al., 2017; Saluzzo et al., 2017), these results suggested that activated ILC2s diverge into two distinct effector fates of pro-inflammatory IL-5/IL-13 producer and tissue-repairing amphiregulin producer ILC2s. Unlike neonatal ILC2s, the divergence between activated adult lung ILC2s KLRG1 and ICOS was not clear. It has been reported that some degree of heterogeneity exist in the adult lung ILC2 population with regard to

cytokine and amphiregulin production after intraperitoneal IL-33 injections (Monticelli et al., 2015). The putative pro-inflammatory KLRG1^{+} ILC2s have high expression of *Arg1* encoding arginase-1 (Arg1), which is required for the cytokine production by ILC2s (Monticelli et al., 2016). KLRG1, an ITIM-bearing inhibitory receptor for E-cadherin, is a marker for highly activated ILC2s and acts as an immune checkpoint (Vivier, 2017; Taylor et al., 2017a). On the other hand, the putative tissue-repairing ILC2s have high expression of ICOS. The ICOS/ligand interaction promotes survival and cytokine production of adult ILC2s after intranasal IL-33 administration (Maazi et al., 2015). In our study ICOS is specifically expressed by amphiregulin-producing neonatal ILC2s. It remains to be determined whether blocking ICOS/ligand interaction inhibits amphiregulin production by these cells. The tissue-repairing ILC2s have high expression of *Tnfaip3*, *Per1* and *Irf2bp2*. *Tnfaip3* and *Per1* encode ubiquitin-modifying enzyme A20 and period circadian regulator 1, respectively, which are known to inhibit NF-κB (Matmati et al., 2011; Sugimoto et al., 2014). *Irf2bp2* encodes interferon regulatory factor 2 binding protein 2, which inhibits NFAT signaling and also acts as an activator of vascular endothelial growth factor A expression in muscle cells (Teng et al., 2010; Carneiro et al., 2011). Therefore, the high expression of these genes suggests that NF-κB and NFAT signaling and cytokine production might be actively inhibited in amphiregulin-producing ILC2s. The tissue-repairing ILC2s also had high expression of *Stab2* encoding stabilin-2. The scavenger receptor stabilin-2 binds to hyaluronic acid and other glycosaminoglycans (Harris et al., 2007; Schledzewski et al., 2011; Harris and Weigel, 2008), which are upregulated in the extracellular matrix during inflammation and has to be taken up and degraded before the original matrix can be restored. The role of Stabilin-2 in promoting tissue repairing phenotype and function of these cells remains to be elucidated.

Overall, neonatal lung ILC2s were divided into two distinct effector subsets and an IL-18Rα^{+}ST2^{-}*Tcf7*$^{+}$ progenitor-like subset. The two effector subsets were defined by the expression of ICOS and KLRG1, and they differentially produced the growth-factor amphiregulin and type 2 cytokines. The IL-18Rα^{+}ST2^{-}*Tcf7*$^{+}$ cells are likely IL-18 responsive lung ILC progenitors, which may contribute to ILC-poiesis in neonatal and inflamed lungs.

Chapter 6: Discussion

Lung ILC2s play important roles in the development of allergic inflammation (Halim et al., 2012a; Martinez-Gonzalez et al., 2015). ILC2 numbers are elevated in the peripheral blood and sputum of asthma patients (Smith et al., 2016; Ying et al., 2016). In addition, ILC2s are involved in other significant lung pathologies including COPD (Silver et al., 2016) and fibrosis (Hams et al., 2014). Hence, they are recognized as potential therapeutic targets. It is important to elucidate the developmental pathways and complexity of the lung ILC2s as well as the functional properties of stimulated ILC2 subsets, so that proper subsets are targeted in therapeutic interventions. During my PhD studies, I explored the developmental pathways and complexity of the lung ILC2s during adult and neonatal periods, using various transgenic mouse models, microarray global gene expression, scRNA-seq, flow cytometric and functional analyses.

During my MSc studies in Dr. Fumio Takei lab, we realized that lung ILC2s actively develop during neonatal period (Ghaedi, 2015; Ghaedi et al., 2016; Steer et al., 2017). We also realized that CLPs were very rare during this period in BM and spleen, whereas LMPPs including LMPP-s and LMPP+s were prevalent. The rare neonatal CLPs, even when transplanted in the same numbers as neonatal LMPP+s, were far less competent than LMPP+s to give rise to ILC2s and T cells. In addition, our and another lab's reports indicated that adult BM LMPPs are more efficient than CLPs in generating ILCs and T cells in transplantation assays (Halim et al., 2012b; Yang et al., 2015b). These results were in contrast to our general understanding that all lymphocytes develop from CLPs and showed similarities with CLP-independent pathways of T cell development (Allman et al., 2003). Hence, I began my PhD studies with addressing the early developmental processes that leads to the generation of ILCs as well as T cells during adulthood, described in Chapter 3. I divided adult BM lymphoid progenitors based on the expression of Kit,

Sca-1, Flt3, CD127 and Ly6D into four populations namely LMPP-s ($Lin^{-}Kit^{hi}Sca\text{-}1^{hi}Flt3^{+}CD127^{-}$), LMPP+s ($Lin^{-}Kit^{hi}Sca\text{-}1^{hi}Flt3^{+}CD127^{+}$), $Ly6D^{-}$ and $Ly6D^{+}$ CLPs ($Lin^{-}Kit^{lo}Sca\text{-}1^{lo}Flt3^{+}CD127^{+}$). I characterized the gene expression profile of these lymphoid progenitors as well as their distinct potentials for different lymphoid lineages in transplantation assays (Ghaedi et al., 2016). I found that LMPP+s were different from conventional LMPP-s, $Ly6D^{-}$ and $Ly6D^{+}$ CLPs in their gene expression profiles as well as their capacity to differentiate into ILCs and T cells. Although, the expression of T cell and ILC-lineage associated genes were generally low among these four populations, LMPP+s were the most efficient progenitor population to engraft thymus and mucosal tissues and generate thymocytes and ILCs, respectively. Interestingly, LMPP+s had residual myeloid potentials as they could generate very small number of myeloid cells early after transplantation and also give rise to granulocyte/monocyte colonies when cultured with IL-3, stem cell factor, IL-6 and erythropoietin for 14 days (Ghaedi, 2015). The Affymetrix microarray global gene expression analysis of LMPP+s provided valuable information with regard to their differential gene expression compared to other lymphoid progenitors. However, we did not detect expression of T cell and ILC-lineage associated genes by LMPP+s. It is unknown whether LMPPs are heterogeneous and only a small subset is committed for the T and ILC lineages. Future studies with scRNA-seq analysis of LMPP+s can provide insights into their heterogeneity and the existence of bi-potent T and ILC as well T or ILC restricted progenitors among them. Also, scRNA-seq analysis of LMPP+s of neonatal spleen and BM as well as adult BM can provide information on how the gene expression profile of LMPP+s changes as a function of time during neonatal and adult periods.

In addition to LMPP+s, CHILPs were also more abundant in the neonatal spleen (Ghaedi et al., 2016) than adult BM (Klose et al., 2014; Gronke et al., 2016; Constantinides et al., 2014,

2015; Yang et al., 2015b). It also became clear that ILCs are tissue resident (Gasteiger et al., 2015). Therefore, we speculated that the contribution of BM lymphoid progenitors in adults to ILC generation in steady state might be limited. Hence, we became interested in investigating the existence of lung-resident ILC progenitors that could replenish ILC2 pool during adulthood. In addition, our analysis of adult RAG/Red mouse lung ILC2s indicated (Ghaedi et al., 2016; Yang et al., 2011) that 40% of ILC2s are RAG/Red^{+} and therefore have developed from *Rag1* positive progenitors, described in Chapter 3. And BrdU labeling of neonatal lung ILC2s indicated that at least 30% of adult lung ILC2s are generated in the neonatal period. These results convinced us that lung ILC2 population is heterogeneous. Therefore, I decided to investigate the existence of tissue-resident ILC progenitors as well as ILC2 heterogeneity and complexity in adult and neonatal lungs, described in Chapters 4 and 5, respectively. In order to do so, I generated RORα lineage tracer mice (RORα-YFP mice). Considering that *Rorα* is expressed in early ILC progenitors (Constantinides et al., 2014; Harly et al., 2017; Ishizuka et al., 2016; Lim et al., 2017a) and mature ILCs (Hoyler et al., 2012; Robinette et al., 2015; Halim et al., 2012b; Wong et al., 2012; Lo et al., 2016), RORα-YFP mice provided a comprehensive model to identify immature and mature lung ILC2s. To further adopt a comprehensive and an unbiased approach for studying ILC2 heterogeneity, we analyzed all adult and neonatal lung CD45$^{lo/+}$Lin^{lo} cells by scRNA-seq and confirmed the results by flow cytometric and functional analyses. In adult RORα-YFP mouse lungs, described in Chapter 4, I found Lin^{-}CD127^{+}Thy1^{+}IL-18Rα^{+}ST2^{-}YFP^{+} cells that expressed low levels of GATA-3, but not T-bet and RORγt. A fraction of these cells expressed *Il5* and *Il13* and upon *in vitro* stimulation with PMA and ionomycin, they could produce the same cytokines as ILC2s, albeit to much lower amounts. Their expression of these cytokines indicated that they belong to the ILC2 lineage. And their lower expression of these cytokines compared to mature

lung ILC2s indicated that they are functionally immature with cells that are perhaps in different degrees of differentiation toward mature ILC2 fate. Intranasal papain treatment resulted in the expansion of these cells, suggesting that they are capable of sensing and responding to tissue inflammation by their expression of IL-18Rα. The Lin^{-}T-$bet^{-}CD127^{+}Thy1^{+}ROR\gamma t^{-}$IL-$18R\alpha^{+}ST2^{-}$ population was also found in *Rag1*$^{-/-}$. These cells also produced lower amounts of ILC2-associated cytokines and expanded after intranasal papain treatment. The scRNA-seq analysis of the IL-$18R\alpha^{+}ST2^{-}$ cells indicated that they express *Tcf7*, and our analysis of *Tcf7*^{EGFP} mice further confirmed this. In addition, the Lin^{-}T-$bet^{-}CD127^{+}Thy1^{+}ROR\gamma t^{-}$IL-$18R\alpha^{+}ST2^{-}$ cells expressed the TCF-1 transcription factor in naïve and papain-treated lungs. IL-$18R\alpha^{+}ST2^{-}$ cells showed close phenotypic similarities to the reported systemic human ILC precursors (Lim et al., 2017a). Both of these populations share the expression of the ILC-lineage associated transcription factor genes *Rora, Tcf7, Gata3, Id2* and *Runx3* as well as *Cd2*, *Cd7, Il7r, Il1r1* and *Il18r1*. They both also have low expression of GATA-3 and lack T-bet and RORγt expression. Consistent with a previous study (Harly et al., 2017) indicating that ILCPs do not depend on CD127 signaling for their development, lung IL-$18R\alpha^{+}ST2^{-}$ cells are also not dependent on CD127 signaling for their development as they are found in naïve and papain-treated RORα-CD127cKO mouse lungs. Hence, the $ST2^{-}$IL-$18R\alpha^{+}$ cells in our study might be ILC progenitors. Albeit *in vivo* and *in vitro* differentiation assays are required to confirm that these cells possess ILC progenitor capacities.

ILC2 development starts soon after birth (Ghaedi et al., 2016; de Kleer et al., 2016; Saluzzo et al., 2017; Steer et al., 2017). Neonatal ILC2s accumulate and expand in the lung and are also activated due to the endogenous IL-33 release by neonatal lung epithelium. Therefore, we also carried the analysis of neonatal RORα-YFP mouse lungs, described in the Chapter 5, which not

only provided us with further insights into their development in the lung during their most active phase of development, but also provided valuable information with regard to the heterogeneity of $ST2^+$ ILC2s. I adopted the same gating strategy that I used for the analysis of adult lung cells, and analyzed all 12-day-old neonatal lung $CD45^{lo/+}Lin^{lo}$ cells by scRNA-seq. By this approach, I identified distinct ILC2 subsets; a *Tcf7* expressing ILC progenitor-like subset as well as distinct *Klrg1*/*Il5*/*Il13* and *Icos*/*Areg* expressing effector ILC2 subsets. The neonatal lung ILC progenitor-like cells are phenotypically similar to BM ILCPs ($Lin^-Thy1^+CD127^+$*Tcf7*$^{GFP+}ST2^-$). In addition, both the ILC progenitor-like cells in neonatal lung and adult BM ILCPs are IL-18Rα^+Kit^+. Hence, they are phenotypically similar to the adult lung $Lin^-Thy1^+CD127^+$IL-18Rα^+ST2^- cells, suggesting that these lung ILC progenitor-like cells might contribute to the ILC development in the neonatal and adult lung.

I divided the neonatal $ST2^+$ ILC2s into two functionally distinct subsets based on the expression of KLRG1 and ICOS. The $KLRG1^+$ subset represented the pro-inflammatory ILC2s and produced large amounts of IL-5 and IL-13. On the other hand, the $ICOS^+$ ILC2s, represented tissue-repairing ILC2s and produced amphiregulin. My results suggested that activated neonatal ILC2s diverge into two distinct effector fates of pro-inflammatory and tissue-repairing ILC2s. In agreement with the pro-inflammatory potential of $KLRG1^+$ ILC2s, these cells have a high expression of *Arg1* encoding arginase-1, which is required for ILC2 cytokine production (Monticelli et al., 2016). On the other hand, the putative tissue-repairing ILC2s have high expression of *Tnfaip3*, *Per1* and *Irf2bp2*, suggesting that NF-κB and NFAT signaling and cytokine production might be actively inhibited in these cells (Matmati et al., 2011; Sugimoto et al., 2014; Teng et al., 2010; Carneiro et al., 2011). I realized that a similar divergence in the expression of KLRG1 and ICOS also occurs with papain-treated adult lung ILC2s. My results are in agreement

with previous reports indicating that IL-33-activated lung ILC2s show some degree of heterogeneity with regard to cytokine and amphiregulin production (Monticelli et al., 2015).

We are currently carrying additional experiments to assess the progenitor properties of $Lin^{-}CD127^{+}Thy1^{+}IL\text{-}18R\alpha^{+}ST2^{-}YFP^{+}$ cells of adult RORα-YFP mouse lungs. In order to do so, I have purified these cells along with BM $Lin^{-}CD127^{+}Thy1^{+}PD\text{-}1^{+}ST2^{-}$ cells (ILCPs) (CD45.2) as controls and transplanted each into lethally irradiated congenic $B6.Ly5^{SJL}$ mice (CD45.1) together with helper BM cells from the lymphocyte-deficient NSG mice (CD45.1). I have also purified *Rag1*$^{-/-}$ mouse lung $Lin^{-}CD127^{+}Thy1^{+}IL\text{-}18R\alpha^{+}ST2^{-}$ cells and BM $Lin^{-}CD127^{+}Thy1^{+}PD\text{-}1^{+}ST2^{-}$ cells (ILCPs) and transplanted each into lethally irradiated congenic B6.Ly5SJL mice (CD45.1). In addition, we will purify the neonatal lung *Tcf7* expressing ILC progenitor-like cells described in our study ($Lin^{-}CD127^{+}Thy1^{+}$*Tcf7*$^{EGFP+}IL\text{-}18R\alpha^{+}ST2^{-}$ cells) from 12-day-old *Tcf7*^{EGFP} mice, along with adult BM $Lin^{-}CD127^{+}Thy1^{+}$*Tcf7*$^{EGFP+}ST2^{-}$ cells (CHILPs and ILCPs) (CD45.2) as controls and transplant each into lethally irradiated congenic $B6.Ly5^{SJL}$ mice (CD45.1). We will analyze the lungs, spleens and intestines of the transplanted mice at 6-weeks post-transplantation to fully access the differentiation potential of adult and neonatal lung $IL\text{-}18R\alpha^{+}ST2^{-}$ cells into different ILC and adaptive lymphoid lineages. We are also assessing the *in vitro* differentiation potentials of the lung $IL\text{-}18R\alpha^{+}ST2^{-}$ cells on stromal cells (OP9-DL1/OP9) with IL-7 and SCF. In addition, we are assessing the proliferation and cytokine capacity of adult lung $IL\text{-}18R\alpha^{+}ST2^{-}$ cells upon IL-18, IL-33 and papain *in vivo* intranasal treatment into RORα-YFP and *Rag1*$^{-/-}$.

We are also investigating the signals that drive the dichotomy between neonatal pro-inflammatory and tissue-repairing ILC2s. Neonatal lung ILC2s show a peak of proliferation and

activation due to the spontaneous release of IL-33 by epithelium (de Kleer et al., 2016; Steer et al., 2017; Saluzzo et al., 2017). Therefore, neonatal lung ILC2 numbers are significantly lower in *Il33*$^{-/-}$ mice than wild type controls (Steer et al., 2017). I am analyzing neonatal *Il33*$^{-/-}$ mouse lung ILC2 for their expression of KLRG1 and ICOS to assess whether IL-33 is responsible for driving the divergence of ILC2 pro-inflammatory and tissue-repairing subsets. Considering that tissue-repairing ILC2s specifically express ICOS, I will assess the roles of ICOS/ligand signaling in amphiregulin production by these cells. To do so, I will purify neonatal lung ILC2s ($Lin^{-}CD127^{+}Thy1^{+}ST2^{+}$) and culture these cells in IL-7+IL-33 in the presence or absence of an ICOS blocking antibody to later assess the amounts of amphiregulin and cytokines in the culture supernatants.

Future Direction

We now know that in addition to IL-18Rα^{+}ST2^{-} ILC progenitor-like cells, multiple mature (ST2^{+}) ILC2 populations co-exist in the adult lung: in the steady state, neonatally-derived ILC2s, adult-derived ILC2s, ILC2s that derive from *Rag1* positive and negative progenitors. I believe that significant avenues of research can follow these studies. One important question is whether ILC2s that are derived during neonatal and adult periods can have different gene expression profiles and functions. Designing proper mouse models can help in answering this question, as an example *Tcf7*-IRES-CreERT2 (Cre recombinase - estrogen receptor T2) mice that are administered tamoxifen over a one month period in adulthood can shed some light on the contribution of adult versus neonatal ILC progenitors to the generation of lung ILC2s and whether adult- versus neonatally-derived ILC2s might be functionally different. Another question that can be asked is whether ILC2s that are generated from *Rag1* positive and negative progenitors might be functionally different. This question can be answered using *Rag1* fate-mapping mouse models. Similar studies on NK cells have indicated NK cells that derive from *Rag1* expressing progenitors are more functionally fit with a higher potential to generate NK memory cells than NK cells that derive from *Rag1* negative progenitors (Karo et al., 2014). It would be interesting to examine the functional fitness and memory generation potential of ILC2s that derive from *Rag1* positive and negative.

Another significant question is how lung ILC2 identity is established and maintained: to what extent cell-intrinsic qualities inherited from their BM precursors versus lung tissue specific signals are important in this process. One possible way to answer this question is to deplete the lung ILC2 pool by irradiation, which also disrupts lung tissue homeostasis, and then compare the gene expression profile of the BM-derived ILC2s, to unperturbed lung ILC2s. Similar studies have

been carried out to compare the gene expression profile of repopulating BM-derived macrophage populations in different tissues and embryonically derived tissue resident macrophages. These studies have indicated that BM derived macrophage populations acquire significantly different transcriptional profiles with much less active tissue-specific enhancers compared to the embryonically derived macrophages (Ginhoux and Guilliams, 2016). In addition, these studies can reveal the tissue specific signals that imprint lung ILC2 identity.

Another significant question is how long-lived neonatal ILC2s self-maintain and persist in adult lungs. Our study also reveals the possibility that lung ILC progenitors might be long-lived and persist from neonatal period to adulthood. Hence the same question might become relevant to tissue-resident ILC progenitors.

Lung ILC2s derive from neonatal and adult ILC progenitors as well as *Rag1* positive and negative progenitors (Ghaedi et al., 2016). These cells show a heterogeneous profile after activation and include $KLRG1^+$ pro-inflammatory and $ICOS^+$ tissue-repairing ILC2s. A subset of activated ILC2s acquire memory-like characteristics and can mediate enhanced responses (Martinez-Gonzalez et al., 2016, 2018). Understanding lung ILC2s origins, developmental pathways and the tissue factors that regulate their identity, maintenance and functional heterogeneity after activation is fundamental for the design of future specific therapeutic interventions.

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