COMMON LYMPHOID PROGENITOR-INDEPENDENT PATHWAYS OF INNATE AND T LYMPHOCYTE DEVELOPMENT

by

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Abstract

All lymphocytes are thought to develop from a single population of committed lymphoid progenitors termed common lymphoid progenitors (CLPs). However, upstream progenitors termed lymphoid-primed multi-potent progenitors (LMPPs) are known to be more efficient than CLPs in differentiating into T cells and group 2 innate lymphoid cells (ILC2s), suggesting alternative pathways of their development. Here, we have divided LMPPs into $CD127^-$ (LMPP-s) and $CD127^+$ (LMPP+s) subsets and compared them with CLPs. Adult LMPP+s are the most efficient progenitors for T cells and ILCs in transplantation assays, and lineage tracking by the recombinase expression also suggests that most ILC2s and NK cells develop from LMPPs independent of CLPs. In the neonatal period CLPs are rare and, unlike prominent neonatal LMPP+s, incapable of differentiating into ILC2s and T cells while their development is highly active. These results suggest non-linear pathways of innate and T lymphocyte development from LMPP+s with limited CLP contributions.

Preface

A part of the introduction is a version of an invited review entitled "Development of Group 2 Innate Lymphoid Cells" by Ghaedi, M., and Takei, F. that is submitted for potential publication.

Chapter 3 of this thesis has been submitted for potential publication: Ghaedi, M., Steer, C., Halim, T., Abraham, N., and Takei, F.: "Common lymphoid progenitor-independent pathways of innate and T lymphocyte development". I carried out all the experiments presented in Chapter 3 with the following exceptions. In Figure 2B, the analysis of CD127-null mice was carried out by Tim Halim. In Figure 5, Catherine Steer provided neonatal mice of different ages and also analyzed lung ILC2s. Over all, for the submitted manuscript, I designed and performed the experiments and wrote the paper. Steer, C. performed the experiments and wrote the paper. Halim, T. performed the experiments and reviewed the paper. Abraham, N. reviewed the paper. Takei, F. supervised the project, designed the experiments 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 number: # A14-0182.

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List of abbreviations

Ab	antibody
APC	allophycocyanin
B6	C57Bl/6
BM	bone marrow
CLP	common lymphoid progenitor
GFP	green fluorescent protein
FACS	florescence activated cell sorting
Flt3L	Fms-like tyrosine kinase ligand
GATA3	GATA-binding protein 3
HSC	haematopoietic stem cell
IFN	interferon
IFNγ	interferon gamma
IL	interleukin
IL-17RB	IL-25 receptor
ILC	innate lymphoid cell

ILC1	innate lymphoid cell group 1
ILC2	innate lymphoid cell group 2
ILC3	innate lymphoid cell group 3
Lin	lineage
LMPP	lymphoid primed multipotent progenitor
LSK	lineage-negative, Sca-1-positive, c-Kit-high
LTi	lymphoid tissue inducing
Ly49	lymphocyte antigen 49 complex
MHC	major histocompatibility complex
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
PI	propidium iodide
RAG	recombinase activating gene

ROR	retinoic acid receptor orphan receptor
Sca-1	stem cell antigen 1
SCF	stem cell factor
TCR	T cell receptor
TGFβ	transforming growth factor-beta
Th	T helper
TNF-α	tumour necrosis factor-alpha
TSLP	thymic stromal lymphopoietin

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Chapter 1 Introduction

1.1 The immune system

The immune system can be divided into adaptive and innate immune systems, each of which is composed of multiple specialized cell types [1]. The adaptive immune system confers a highly antigen specific response through recombination activating gene (RAG) mediated V(D)J recombination and generation of receptors of diverse specificities. This compartment of the immune system consists of T and B lymphoid lineages [2]. T cells are generated in the thymus and they can be divided into two types based on the T cell receptor (TCR) expression, namely TCRαβ- and TCRγδ-expressing T cells. The latter population mostly develops in the perinatal period and seeds various tissues. Adult T cells are predominantly TCRαβ$^+$ and they are further sub-grouped to CD8$^+$ cytotoxic T cells and CD4$^+$ T helper (Th) cells. CD8$^+$ cytotoxic T cells destroy infected or transformed cells [2] whereas CD4$^+$ T helper (Th) cells secrete cytokines that influence (stimulate) other immune functions. Th cells can be further divided into distinct subsets based on the cytokines they produce, namely Th1, 2, 9, 17 and follicular Th subsets. T cells also include regulatory T cells that inhibit other lymphocytes and Natural Killer T (NKT) cells which share properties with innate Natural Killer (NK) cells [3], [4]. B cells are generated in the bone marrow (BM) and confer antibody mediated humoral immunity [2]. They express cell surface Ig, CD19, CD20 and B220 and can be divided into B2 (classical B cells) and B1 subsets, based on their differences in genetical program, function and anatomical location. B1 cell subset is an innate-like B cell population and source of innate-Abs that is protective against commonly encountered pathogens. B1 cells are also a source of Abs directed against self-antigens that play an important role in homeostasis [5].

The innate immune system provides a primary line of defence, and is mediated by non-lymphoid cells including granulocytes, monocytes and mast cells as well as innate lymphoid cells (ILCs). Identification of several subsets of the ILCs over the past few years has further revealed the significant contribution of the innate immune system in immune regulation.

1.2 ILCs

ILCs, that lack the expression of antigen-specific receptors and can develop in RAG-deficient mice, are mainly found at mucosal barriers and respond to factors derived from the epithelium in an antigen non-specific manner. ILCs are important in the regulation of tissue homeostasis and protection against pathogens [6]. The ILC family includes two separate types, cytotoxic and cytokine-producing ILCs [7].

1.2.1 Cytotoxic ILCs

Cytotoxic ILCs are conventional NK cells that resemble the adaptive cytotoxic $CD8^+$ T cells. NK cells express NK1.1 (in C57BL/6 mice), NK cell p46-related protein (NKp46), class I major histocompatibility complex (MHC)-specific inhibitory molecules (NKG2A and Ly49 receptors) and the stimulatory receptor NKG2D. They express the T-box transcription factors Eomes and T-bet and require Eomes, but not T-bet, for their development. They respond to the stimulating cytokines interleukin-18 (IL-18), IL-12 and IL-15 and are dependent on IL-15 for their development and maintenance. NK cells express granzymes and perforin that mediate cell contact dependent cytotoxicity. They also produce interferon-γ (IFN-γ) and tumor necrosis factor (TNF) and are involved in the tumor immunosurveillance and protection against viral infections [8], [9].

1.2.2 Cytokine-producing ILCs

Cytokine-producing ILCs are divided into three subsets, namely group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3), based on the expression of distinct transcription factors and cytokines parallel to that of helper CD4+ T cells (Table A-1). LC1s are similar to NK cells. They express the NK cell receptors NK1.1 and NKp46 as well as the transcription factor T-bet, but not Eomes. T-bet is required for their development. ILC1s like NK cells respond to IL-12 and IL-15 and produce IFNγ and TNFα, but IL-15 is not required for their development or maintenance. ILC1s are less cytotoxic and produce more cytokines than NK cells. ILC1s were initially considered to be a subset of NK cells. However, the finding of the distinct developmental requirements of ILC1s has led to their re-classification as one of the cytokine-producing ILC lineages different from the cytotoxic NK cell lineage. Intestinal ILC1s are involved in the protection against the intracellular protozoan *Toxoplasma gondii* [10]. Based on the expression of the transcription factor T-bet and the production of the type 1 cytokines (IFN-γ and TNF), ILC1s are considered to be the innate counterparts of the adaptive T helper 1 (Th1) cells [7], [10].

ILC2s are the innate counterparts of the adaptive Th2 cells as both express the transcription factor GATA-3 and produce IL-5 and IL-13 [11]. Characterization, function and development of ILC2s are discussed in detail below.

ILC3s include two distinct subsets, $CCR6^{hi}$ lymphoid tissue inducer (LTi) cells that emerge prenatally and are important for the development of lymph nodes, Peyer's patches and intestinal lymphoid clusters, and $CCR6^{lo/-}$ ILC3s that emerge during the first 2-4 weeks after birth and are dependent on the signals from the aryl hydrocarbon receptor (AHR), a transcription factor activated by the environmental toxins, for their expansion. All ILC3s

express the transcription factor RORγt, on which they are dependent for their development, and produce IL-22 and IL-17. ILC3s are the innate counterparts of Th17 cells. IL-22 produced by ILC3s is involved in the defense against attaching-and-effacing intestinal pathogens such as *Citrobacter rodentium* [7], [12].

1.2.2.1 Characterization of ILC2s

ILC2s are activated by the epithelial cell-derived IL-25, IL-33 or thymic stromal lymphopoietin (TSLP) and produce the signature type 2 cytokines IL-5 and IL-13. They are involved in the immunity against helminth infections and also implicated in the allergic airway inflammation [13]. The existence of ILC2s was first reported after the discovery that IL-25 administration induces IL-5 and IL-13 production in RAG2 deficient mice that lack B and T cells [14]. Subsequently, a similar population was reported to provide a crucial source of type 2 cytokines in the initial stages of helminth infection [15]–[18]. Later on ILC2s were further characterized and shown to be present in the lungs, intestines, liver, spleen, lymph nodes and fat-associated lymphoid clusters (FALC) [19]–[24]. ILC2s were also shown to be important in the lung epithelial repair after influenza virus infection as they produce amphiregulin, a member of the Epidermal Growth Factor (EGF) family of growth factors [25], and in the initiation of type 2 adaptive immunity [26], [27]. ILC2s, like other ILCs, lack the expression of cell surface markers for mature hematopoietic and lymphoid cells (Lin^-). They express CD127 (IL-7Rα), CD25 (IL-2Rα) and the receptors for the cytokines IL-33 (ST2) and IL-25 (IL-17RB). Thus, mouse ILC2s are defined by $Lin^-Id2^+IL7R\alpha^+Thy1^+CD25^+ST2^{+/-}Sca\text{-}1^+IL\text{-}17RB^+$ [28]. Human ILC2s, identified by $Lin^-IL\text{-}7Ra^+CD25^+CD161^+CRTH2^+$, have been found in the lung, intestinal tissues and in elevated numbers in the nasal polyps of the patients with chronic rhinosinusitis [29].

1.3 Lymphopoiesis

Mature blood cells derive from haematopoietic stem cells (HSC) that are located in the BM. HSCs that are multipotent and have self-renewal ability, differentiate into all types of blood cells via a stepwise loss of lineage potentials. During this process, HSCs lose their self-renewal capacity and differentiate into multipotent progenitors. HSCs are identified in mice as a rare population among the BM $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{hi}Flt3^{-}$. These cells are further defined as $EPCR^{+}CD48^{-}CD150^{+}$ (ESLAM) cells (Benz et al. 2012). HSCs differentiate into common myeloid progenitors (CMPs) and lymphoid primed multipotent progenitors (LMPPs). CMPs that are defined as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}CD34^{+}CD16/32^{+}$ BM cells give rise to granulocyte/macrophage and megakaryocyte/erythrocyte progenitors. LMPPs that are defined as $Lin^{-}cKit^{hi}Sca\text{-}1^{hi}Flt3^{hi}CD127^{-}$ BM cells represent a population of progenitors that are primed for the lymphoid lineage development but retain residual myeloid potential [31].

In the classical model of lymphopoiesis LMPPs give rise to common lymphoid progenitors (CLPs) and all lymphocytes develop from the CLPs in the BM [32]. They lack any myeloid potential and equally give rise to all lymphocytes including T, B and NK cells. CLPs are functionally defined by their clonal output of all lymphoid lineages in in vitro cultures and transplantation assays. They were initially identified by a combination of cell surface markers, namely Lineage marker-negative (Lin^{-}) $cKit^{lo}Sca\text{-}1^{lo}CD127^{hi}$ (IL-7Rα^{hi}), which has been refined to include other markers, including high expression of Fms-related typrosin kinase 3 (Flt3, CD135) [33]. In the classical model, CLPs are the obligatory intermediate progenitors between LMPPs and any mature lymphocytes. However, the discovery and characterization of early thymic progenitors (ETPs) suggested that T cell development might not follow this classical pathway. ETPs were identified as the most immature and efficient T cell progenitors in the thymus. ETPs have residual myeloid

potential and their development is mostly independent of the transcription factor Ikaros that is required for the development of CLPs, suggesting that ETPs can develop via a CLP-independent pathway [34], [35]. Furthermore, LMPPs or a subset of LMPPs termed Early Lymphoid Progenitors (ELPs) that was defined by the expression of recombination-activating gene 1 (*Rag1*) are much more efficient than CLPs in differentiating into T cells in transplantation assays [36]. It is evident that CLPs have the potential to differentiate into T cells, but they are barely detectable in the peripheral blood and seem to mostly remain in the BM. In contrast, circulating LMPPs can readily be detected [37]. The T cell potential of CLPs may be realized only when they are isolated from the BM and transplanted into irradiated recipients. A compelling amount of evidence supports a CLP-independent pathway of T cell development in normal steady state.

1.4 The development of ILCs

1.4.1 BM NK progenitor (NKP)

The first stage to the generation of NK cells occurs when lymphoid progenitors differentiate into NK progenitors (NKPs). According to the classical model, the lymphoid progenitors upstream of NKPs are CLPs. NKPs have acquired the IL-15 receptor complex, composed of IL-15 receptor α (IL-15Rα), IL-2Rβ (CD122), and IL-2 receptor common γ (IL-2Rγc), and are responsive to IL-15, which is required for NK cell development and survival. NKP has lost the ability to differentiate into T cells, B cells. These cells first acquire NK1.1 and NKG2D expression, and later acquire other NK lineage markers such as Ly49 family members and DX5 and become functionally mature [38]–[41].

1.4.2 BM ILC2 progenitors

Adult BM contains ILC2-like cells [42], [43]. They were shown to be closely related to

ILC2s by the genome-wide transcriptome analysis, have very low levels of cytokine production upon *in vitro* stimulation with IL-33 and TSLP and were suspected to be the lineage-specified precursors to ILC2s. Upon transplantation into alymphoid *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice, they only gave rise to mature ILC2s in the mucosal tissues such as lungs and intestines and were subsequently named ILC2 progenitors (ILC2Ps). ILC2Ps are defined by Lin^{-}Id2^{+}$\alpha_4\beta_7^{+}$IL-7Rα^{+}Thy1^{+}CD25^{+}ST2^{+} (Hoyler et al. 2012; Halim et al. 2012).

1.4.3 Common developmental programs to all ILCs and a common progenitor to all cytokine-producing ILCs

ILCs are thought to develop from CLPs (defined by Lin^{-}cKit^{lo}Sca-1^{lo}Flt3^{hi}IL-7Rα^{+} that also give rise to T and B cells [45]. All ILCs share the requirement for the transcriptional repressor, inhibitor of DNA-binding 2 (ID2) for their development (Diefenbach et al. 2014; Benz et al. 2012). ID2 is a member of the Helix-Loop-Helix (HLH) family of transcriptional regulators, which lacks DNA binding domain and its hetero-dimerization with E proteins inhibits DNA binding of E proteins and represses their transcriptional activity. E proteins belong to the class I basic HLH (bHLH) family of transcription factors and are strictly required in the T and B cell development. ID2 suppression of the T and B cell fates is crucial in the development of all ILCs and mice lacking this transcription factor lack all ILCs [47]. Notch and IL-7 signaling pathways are also required for the development of cytokine-producing ILCs [7]. The shared developmental requirements of ILCs, led to the speculations that an ID2-expressing common progenitor to all or some ILCs should exist [11]. Examination of the fetal liver and adult BM of the ID2 reporter mice, in which green fluorescent reporter protein is expressed under the control of the *Id2* promoter and ID2-expressing cells are readily identified, confirmed the existence of such an ID2-expressing progenitor. Upon transfer into alymphoid mice, the ID2^{+} progenitors gave rise to ILC1 (T-

bet^{+}Eomes^{-}), ILC2 (GATA-3^{+}), ILC3 (RORγt^{+} including LTi cells), but not to NK (T-bet^{+}Eomes^{+}), T or B cells [10]. Therefore, the ID2^{+} progenitors were committed common progenitors for all the cytokine-producing (helper-like) ILCs and were named CHILPs (common progenitors to all helper-like ILCs). CHILPs are Lin^{-}Id2^{+}PLZF$^{+/-}$α4β7^{+}IL-7Ra^{+}Thy1^{+}Flt3^{-}CD25^{-}ST2^{-}. The expression of ID2 and α4β7 and the lack of Flt3 expression distinguish CHILPs from CLPs. A subset of CHILPs expresses the transcription factor promyelocytic leukemia zinc finger (PLZF), encoded by the *Zbtb16* gene. PLZF is required for the development of NKT cells and confers their innate properties [48], [49]. PLZF^{+} CHILPs highly express *Gata3*, *Rora*, *Tcf7* (encoding TCF-1) and low level of *Rorct* (encoding RORγt). While ILCs do not express PLZF, lineage tracing by the expression of *Zbtb16*, a genetic fate mapping mouse model, in which Cre recombinase is expressed under the control of *Zbtb16* promoter and permanently activates the yellow fluorescent reporter protein (YFP), has revealed that cytokine-producing ILCs, but not NK cells or LTi, show a history of substantial *Zbtb16* expression during their development. In agreement with the lineage tracing data, PLZF^{+} CHILPs had the potential to give rise to ILC1, ILC2 and ILC3s but not to NK or LTi cells upon transfer to alymphoid mice [45], showing their restricted developmental potential in comparison with CHILPs. As PLZF transcription is thought to be controlled by ID2 [50], the PLZF^{+} CHILPs might be the downstream progeny of the PLZF^{-} CHILPs (Figure 1).

Compelling evidences indicate that NK cell specification differ from cytokine-producing ILC specification. NK cells are not the downstream progeny of CHILPs [10], [45], NKPs are mainly ID2^{-} and ID2 is only required after their lineage specification and further differentiation into NK cells [41], [51]. It has also been shown that while GATA-3 is required for the development of all cytokine-producing ILCs, it is dispensable for the

development of NK cells [7]. Therefore, NK cells are now considered to constitute the lineage of cytotoxic ILCs separate from all the cytokine-producing ILC lineages.

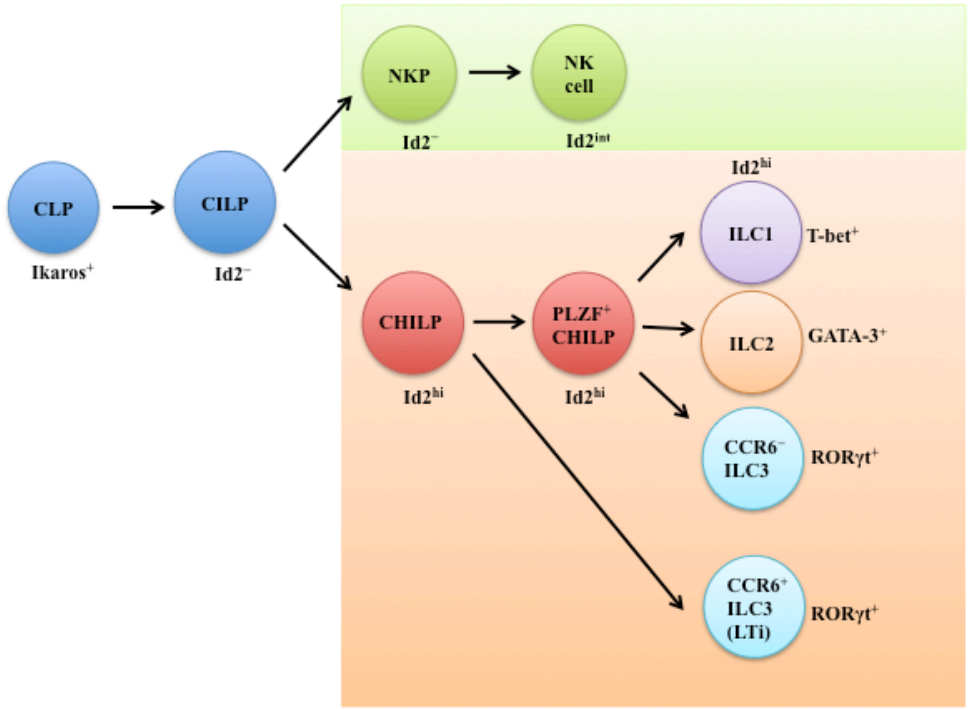

Figure 1 Lineage map for the development of cytotoxic and cytokine-producing ILCs.

All lymphoid lineages are thought to be the downstream progenies of CLPs. The existence of an ILC-restricted progenitor (CILP) to the two main ILC lineages, cytotoxic and cytokine-producing ILCs, is speculated. NK cells are the only cytotoxic ILCs whereas cytokine-producing ILCs are composed of ILC1s, ILC2s and ILC3s. All cytokine-producing ILCs (but not cytotoxic ILCs) differentiate from the CHILPs. A subset of CHILPs is $PLZF^{+}$ and has more restricted developmental potential in compare to CHILPs. $PLZF^{+}$ CHILPs have the potential to give rise to ILC1, ILC2 and ILC3s but not to LTi cells. CLP, common lymphoid progenitor; CILP, a speculated common ILC progenitor of both cytotoxic ILCs and cytokine-producing ILCs; CHILP, common helper-like ILC progenitor; NKP, NK- restricted progenitor.

1.4.3.1 All ILCs require the transcription factor, nuclear factor interleukin-3 (IL-3) regulated (NFIL3) for their development

Previously it was shown that the differentiation of NK cells requires the transcription factor

NFIL3 (also known as E4BP4) [52], [53] . One recent report has shown that *Nfil3*$^{-/-}$ mice lack CHILPs and have reduced numbers of ILC2s and ILC3s. These mice were also compromised in immune responses related to ILC subsets [54], [55]. Suggesting that NFIL3 might be required for the generation of both cytotoxic and cytokine-producing ILCs.

1.4.4 Transcription factors regulating ILC2 differentiation

CHILPs are directed toward the specific ILC lineages by the expression of the lineage-specifying transcription factors. RORα, GATA-3, TCF-1 and growth factor independent 1 (Gfi1) have been shown to be the key transcription factors in ILC2 development, maintenance and specification.

1.4.4.1 RORα

ILC2s highly express the transcription factor RORα (Table A-2), a member of the retinoic-acid-receptor-related orphan nuclear receptor (ROR) family, which also include the transcription factor RORγt. Using the Rora mutant staggerer (Rorαsg/sg) mice that are functionally RORα-deficient due to a spontaneous deletion in the DNA-binding domain of RORα, our lab demonstrated that RORα is specifically required for the ILC2 development. Although Rorα transcript is expressed to a lower degree in other ILC populations [56], RORα-deficiency specifically leads to ILC2 deficiency and does not affect the frequencies of other ILCs or adaptive lymphocytes (Halim et al. 2012). A genome-wide association study comparing asthmatic patients with healthy individuals showed that single-nucleotide polymorphisms (SNPs) within the Rorα gene were associated with asthma [57]. RORα-deficient mice also failed to develop allergic asthma [58]. Therefore, RORα may be a potential target for therapeutic interventions in allergy and asthma.

1.4.4.2 GATA-3

The double zinc-finger transcription factor GATA-3 is required for the transcriptional regulation of the genes encoding the signature Th2 cytokines including *Il5* and *Il13*, and Th2 differentiation of naïve $CD4^+$ T cells [59]. *Gata3* deletion resulted in impaired development of both Th1 and Th2 cells, but not that of $CD8^+$ T cells [60]. Recently, several groups have shown that ILC2Ps in the BM and mature ILC2s in the mucosal surfaces express high amounts of GATA-3 [19], [21], [43]. Mature ILC2s required GATA-3 for the production of type 2 cytokines after *N. brasiliensis* infection in mice [61], and human ILC2s were shown to require GATA-3 for their cytokine production *in vitro* [56]. It should be noted that *Gata3* deletion early in all hematopoietic cells blocked the development of all cytokine-producing ILCs but not cytotoxic ILCs (NK cells) [62]. However, *Gata3* deletion in all ILCs (all $Id2^+$ cells) led to the selective loss of ILC2Ps in the BM and mature ILC2s *in vivo* while other ILCs developed normally [21]. Therefore, the role of GATA-3 in ILC development and function is complex and stage-specific, resembling that in T cell development.

1.4.4.3 Notch and TCF-1

ILC2 development *in vitro* requires Notch signalling [63]. Retroviral transduction of dominant-negative Mastermind like-1 (dnMAML), a pan-Notch inhibitor, into BM multipotent progenitors resulted in impaired ILC2 development *in vivo* [64], suggesting that Notch signaling is also required for the ILC2 development *in vivo*.

The high mobility group (HMG)-box transcription factor TCF-1 is essential in T cell lineage specification. TCF-1, encoded by the *Tcf7* gene, is directly up-regulated by Notch signaling during T cell development [65]. Lung ILC2s express *Tcf7*, and $Tcf7^{-/-}$ mice lack ILC2Ps in the BM and mature ILC2s in mucosal barriers, suggesting that TCF-1 is required

for ILC2 development. Also *Tcf7*$^{-/-}$ mice were compromised in immune responses to the nematode *N. brasiliensis* and protease allergen papain that requires ILC2s [64]. Interestingly, transduction of TCF-1 into dnMAML-expressing BM progenitors partially restored ILC2 generation from the progenitors, indicating that Notch regulation of ILC2 development is at least in part TCF-1 dependent [64]. Notch signaling pathway and the downstream TCF-1 in ILC2 development resemble that of T cell development. TCF-1 up-regulates the expression of *Il17rb* and *Il2ra* through up-regulation of *Gata3*, and directly up-regulate the expression of *Il7r* in ILC2s through binding to a conserved binding site upstream its transcription initiation site [64]. Notch signaling is also required for the development of ILC1s and ILC3s [7]. It was also shown that *Tcf7*$^{-/-}$ mice have reduced numbers of ILC3s [64].

1.4.4.4 Gfi1

The transcription factor Gfi1 has broad roles in different hematopoietic lineages [66]–[69]. One of the roles of Gfi1 is the reciprocal regulation of Th2 and Th17 cell fates by promoting Th2 and antagonizing Th17 polarization in naïve T cells undergoing Th2 differentiation [70]. Similarly Gfi1 is required for the proper specification of ILC2s. Gfi1-deficient ILC2s had impaired GATA-3 expression, and expressed *Rorct* and *Il17a* and the cytokine receptors IL-23R and TGF-βR3 that promote the differentiation of ILC3s. It is notable that the ILC3 lineage is the innate counterpart of the adaptive Th17 lineage. In addition to the suppression of the ILC3 lineage, Gfi1 directly targets and activates *Il1rl1* gene encoding the IL-33 receptor (ST2). Accordingly, Gfi1-deficient mice were impaired in mounting an effective type 2 immunity to *N.brasiliensis* infection and allergic inflammation in response to the protease allergen papain [71].

1.4.5 CHILP and ILC development

According to the classical model the newly found ILCs and CHILPs are considered to develop from CLPs, however the upstream developmental processes of CHILPs have never been elucidated. ILCs can be generated from CLPs [45], [63]. However, we and others have previously reported that LMPPs are much more efficient than CLPs in generating BM ILC2 Progenitors (ILC2Ps) and lung ILC2s in transplantation assays (Yang et al. 2011; Halim et al. 2012). To further elucidate the differentiation pathways of ILCs, we now have extended our studies and characterized various lymphoid progenitors in both adult and neonatal mice and compared their capacities to differentiate into ILCs and other lymphoid lineages. Our results suggest that most T cells, CHILPs and ILCs develop from a subset of LMPPs defined by CD127 expression via CLP-independent pathways. This indicates that the CLP stage is not compulsory for the development of all lymphocytes. Based on our findings, we propose an alternative model of lymphocyte development, in which lymphoid progenitors upstream of CLPs have direct contributions to the production of T cells and ILCs.

1.5 Thesis objectives

The overall objective of this thesis is to elucidate the developmental processes of ILCs and CHILPs, common progenitors of all ILCs. Our first goal is to determine if all lymphocytes develop from CLPs and if there are CLP-independent developmental pathways for innate lymphocytes. We characterize lymphoid progenitors in the BM and their potential for ILCs as well as other lymphocytes by transplantation assays, in vitro differentiation cultures and in vivo lineage tracing analysis. Our second goal is to characterize lymphoid progenitors and their development into ILC2s and T cells in neonatal mice. We analyze different lymphoid progenitors in neonatal hematopoietic organs including spleen and BM and investigate their capacities to differentiate into ILC2s and T cells.

Chapter 2 Materials and methods

2.1 Mice

C57BL/6 (B6), B6.Ly5^{SJL} and NOD.SCID.*Il2rg*$^{-/-}$ (NSG) mice were maintained in the British Columbia Cancer Research Centre (BCCRC) animal facility, under specific pathogen free conditions. B6.129P2(Cg)-*Rorc*^{tm2Litt}/J and B6.129S(Cg)-*Id*$^{2tm2.1Blh}$/ZhuJ (Id2$^{Gfp/+}$) mice were purchased from the Jackson Laboratories. C57BL/6-*flt3Ltm1Imx* mice were purchased from Taconic Farms. Adult mice were used at 6-8 weeks of age. 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.

2.2 BM transplantation

B6.Ly5^{SJL} (CD45.1) mice were lethally irradiated (10 Gy) and intravenously transplanted with highly purified progenitors from C57BL/6 mice (CD45.2) and 10^5 helper BM cells from NSG mice (CD45.1). Drinking water of the irradiated mice was supplemented with Ciprofloxacin and HCl for 4 weeks.

2.3 Rag-1/Red and Rag-1/GFP

BM from Rag-1/Red and Rag-1/GFP was received from Dr. Paul Kincade's laboratory, Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center. Cell suspensions were prepared from the BMs and analyzed by flow cytometry. Also 5-8×10^6 BM cells were intravenously transplanted into lethally irradiated (10 Gy) B6.Ly5^{SJL}. Transplanted mice were used after 2 months for analysis.

2.4 Primary leukocyte preparation

Cell suspensions were prepared from the BM, lungs and spleen as described [73]. Cell

suspensions from small and large intestines were prepared as described (Halim et al. 2012).

2.5 Flow cytometry and cell sorting

All antibodies were purchased from eBioscience unless otherwise specified. Lineage (Lin) marker mABs conjugated with either FITC or eFluor® 450 included anti-CD3ε, TCRαβ, TCRγδ, CD19, B220, NK1.1, Mac-1, Gr-1, Ter119 and CD11c. Additional Abs used included FITC-conjugated anti-CD45.1, PE-conjugated anti-CD127, PerCP-Cy5.5-conjugated anti-CD19, CD25, ST2, PE.Cy7-conjugated anti-Sca-1, PE.Cy5-conjugated anti-CD25, allophycocyanin (APC)-conjugated anti-CD117 (cKit), NK1.1, APC-eFluor780®-conjugated anti-B220, Alexa Fluor® 700-conjugated anti-CD45.1, and eFluor® 605NC-conjugated Thy1.2. BD Horizon V500-conjugated anti-CD45 and CD45.2 were purchased from BD Bioscience. FITC-conjugated anti-ST2 was purchased from MD Bioproducts. Propidium iodide (PI) or eFluor® 780 (eBioscience) were used to exclude non-viable cells. The BD LSRFortessa™ cell analyzer was used for phenotypic analysis; BD FACSAria III was used for cell sorting. Flowjo v. 8.7 (Treestar) was used for data analysis.

2.6 RNA isolation and microarray

Total RNA from highly purified sorted progenitors was extracted using Trizol (Invitrogen). 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 Methylcellulose culture

Sorted progenitor cells (1,000 cells per plate) were cultured in Iscove's MDM-based methylcellulose medium (MethoCult GF 3434; StemCell Technologies) supplemented with

10 ng/ml of recombinant mouse (rm) IL-3, 50 ng/ml of rm stem cell factor, 10 ng/ml of recombinant human (rh) IL-6, and 3 units of rh erythropoietin to support the growth of BFU-E, CFU-GM, and CFU-GEMM. After 2 weeks, colonies were counted and classified.

2.8 Limiting dilution cultures for in vitro T cell differentiation

Limiting dilution assays were performed by culturing CLPs, LMPP-s and LMPP+s in serial dilutions (30–1 cells/well) on OP9-DL1 cells for three weeks in complete RPMI and in the presence of IL-7, and SCF (20 ng/ml each), after which they were analyzed by flow cytometry.

2.9 Accession numbers

The microarray data is submitted to the Gene Expression Omnibus (GEO) database and the accession number is to be obtained.

2.10 Statistics

Data were analyzed using GraphPad Prism version 6 (GraphPad Software). A Student's t test was used to determine statistical significance between groups.

Chapter3 Results

3.1 LMPPs in adult mouse BM can be divided into two subsets by CD127 expression

We first divided Lin^- cells in adult mouse BM into subpopulations based on the expression of cKit and Sca-1. Most $cKit^{hi}Sca\text{-}1^{hi}$ (LSK) cells (Figure 2, red gate) did not express Thy1 (CD90) or ST2 (IL-33R) and contained the conventional LMPPs defined by high levels of Flt3 expression. Interestingly a minor fraction of LMPPs was positively stained for CD127. CLPs were found in the $cKit^{lo}Sca\text{-}1^{lo}$ population (blue gate), which were mostly $Thy1^-ST2^-$, as $Flt3^{hi}CD127^+$ cells, whereas ILC2Ps (Halim et al. 2012) were $cKit^{lo/-}Sca1^{hi}$ (green gate), expressed high levels of Thy1, ST2 and CD127 but were negative for Flt3. In addition, $cKit^-$ cells included a population of $Sca1^{lo/-}$ cells (yellow gate), often termed LSK- cells [74], [75]. This population had very little capacity to differentiate into lymphocytes in transplantation assays (data not shown) and was not further analyzed in this study. To confirm the expression of CD127 on LMPPs, we stained LMPPs from wild type (WT) and CD127-null mice with anti-CD127. Flow cytometric analysis showed clear expression of CD127 on a subset of WT LMPPs (Figure 2B). Therefore, LMPPs were divided into the $CD127^-$ and $CD127^+$ subsets and termed LMPP- and LMPP+, respectively, hereafter.

ELPs are previously characterized $Rag1^+$ subset of the adult mouse BM LMPPs. As ELPs were reported to be $CD127^-$, we examined *Rag1* expression in LMPP-s and LMPP+s in Rag1-GFP knock-in mice [36]. Whereas the majority of Lin^-GFP^+ cells were found in the $cKit^{lo}$ population (Figure 2C, top left, blue gate), the $cKit^{hi}$ population also expressed GFP albeit at lower levels and lower frequencies (red gate). When the LMPP population was divided into $CD127^-$ and $CD127^+$, most GFP^+ cells were in the latter while only a small fraction of the former expressed GFP at a low level. Most CLPs highly expressed GFP.

We further compared LMPP- and LMPP+ by Affymetrix microarray analysis (Figure 2D). The expression of the lymphoid lineage-associated genes, including *Il7r, Ets1, Rag1, Dntt* and *Lck* were higher in LMPP+ than LMPP-. LMPP+ also highly expressed the myeloid cell lineage-associated genes *Mpo*, *Fcer1g*, *Ctsg*, *Fes* and *Prtn3* although the expression of myeloid genes was generally lower in LMPP+ than LMPP-. Both populations expressed the key transcription factors Ikaros (*Ikzf1*), PU.1 (*Sfpi1* in mouse), and E protein family member E2A (*Tcf3*) required for early lymphocyte developmental programs [76] while neither populations expressed the signature genes of the earliest T (*Ptcra*) and B (*Pax5*) cell restricted progenitors (Figure A-1). In addition, the expression of the gene encoding the chemokine receptor CCR9 that is important for trafficking into the thymus [35] was significantly higher in LMPP+s than LMPP-s. The expression of GFP by LMPP+s and their distinct gene profiles compared to LMPP-s support the notion that LMPP-s and LMPP+s are two distinct subsets.

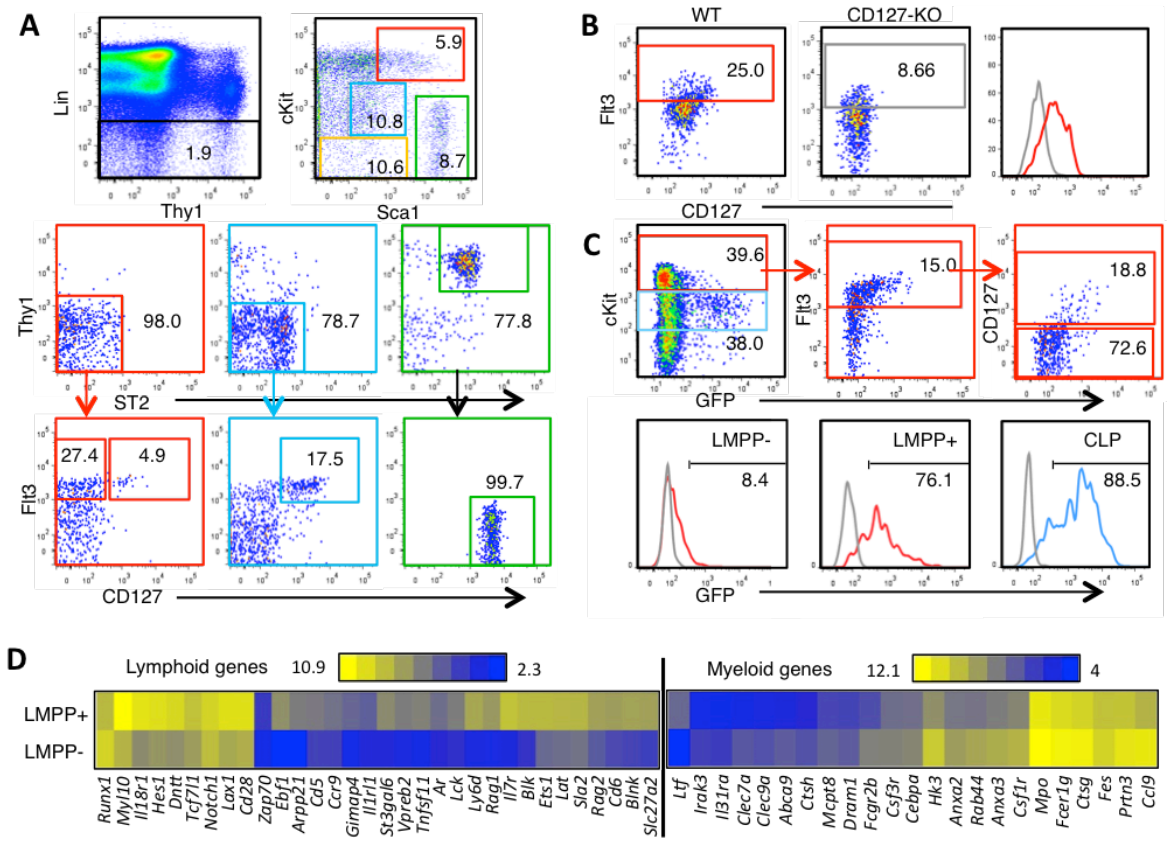

Figure 2 Adult mouse BM LMPPs can be divided into LMPP- and LMPP+.

(A) Adult B6 mouse BM cells were analyzed by flow cytometry. Live (PI^-) $CD45^+$ cells were gated for Lin^- (black gate) and then for $cKit^{hi}Sca\text{-}1^+$ (red gate), $cKit^{lo}Sca\text{-}1^{lo}$ (blue gate), $cKit^-Sca1^{lo/-}$ (yellow gate) and $cKit^{lo/-}Sca1^{hi}$ (green gate). $CKit^{hi}Sca\text{-}1^+$ cells were further gated for $Thy1^-ST2^-$ (red gate, middle left) and divided into $Flt3^{hi}$ $CD127^-$ LMPPs (LMPP-s) and $Flt3^{hi}CD127^+$ (LMPP+s) (bottom left). $CKit^{lo}Sca\text{-}1^{lo}$ cells were gated for $Thy1^-ST2^-$ (blue gate, middle center) and $Flt3^{hi}CD127^+$ (CLPs) (bottom center). $CKit^{lo/-}Sca1^{hi}$ were gated for $Thy1^+ST2^+$ (green gate, middle right) and $Flt3^-CD127^+$ (ILC2Ps) (bottom right). Numbers in plots indicate mean percentages of the gated cells in each plot.

(B) BM cells from WT and CD127-null mice were analyzed by flow cytometry. Lin^- $cKit^{hi}$ $Sca\text{-}1^+$ cells were first gated and the expression of Flt3 and CD127 were analyzed. The expression of CD127 on WT (red) and CD127-null (grey) LMPPs were compared.

(C) $Lin^-cKit^{hi}Sca\text{-}1^+$ (red gate) and $cKit^{lo}Sca\text{-}1^{lo}$ cells (blue gate) from adult RAG-1/GFP mouse BM were analyzed for GFP expression by flow cytometry (top left). The $cKit^{hi}$ population was divided by Flt3 expression and the $Flt3^{hi}$ subset (red gate, top middle) was subdivided by CD127 expression into LMPP-s and LMPP+s (top right). Bottom histograms show the expression of GFP by LMPP-s (red), LMPP+s (red) and CLPs (blue) from RAG-1/GFP and WT control (gray).

(D) RNA was extracted from purified LMPP-s and LMPP+s and analyzed for global gene expression by Affymetrix microarray. The expression of the genes associated with lymphoid and myeloid lineages in LMPP+s was compared to those of LMPP-s in triplicate experiments and shown in heat maps.

The results are representative of more than 6 experiments for A, 4 experiments for B, and 3 experiments for C. See also Figure A-1.

3.2 Adult BM LMPP+s are potent progenitors for T cells and ILCs.

To determine the differentiation capacities of LMPPs, we purified LMPP-s, LMPP+s and CLPs from adult C57BL/6 mouse BM (CD45.2) by cell sorting and transplanted 2,000 cells each into lethally irradiated congenic B6.Ly5^{SJL} mice (CD45.1) together with helper BM cells from the lymphocyte-deficient NOD.SCID.*Il2rg*$^{-/-}$ (NSG) mice (CD45.1). LMPP+ transplantation resulted in strikingly robust thymic engraftment, and much higher numbers of thymocytes were generated from LMPP+s than LMPP-s and CLPs 3 weeks post-transplantation (Figure 3A). We also investigated their in vitro T cell differentiation by limiting dilution cultures on OP9-DL1 stroma cells [77]. CD4$^+$ and CD8$^+$ double positive T cells were generated from the plated progenitor populations after three weeks. The frequency of the cells that could generate CD4$^+$ and CD8$^+$ double positive T cells was not significantly different among the three progenitor populations (Figure A-2A). These results suggested that the efficient thymus engraftment of LMPP+s might be due to their ability to circulate and settle in the thymus. It was reported that BM LMPPs were the main T lymphoid progenitors that have access to the thymus through the blood [34], [37]. Therefore, we analyzed the progenitors in the peripheral blood and found that the circulating LMPPs were uniformly CD127$^+$, phenotypically resembling BM LMPP+s (Figure A-2B).

In contrast to T cells, the numbers of spleen B cells (CD19$^+$B220$^+$) derived from the three donor progenitor populations were not significantly different at 3 weeks post

transplantation (Figure 3B). At one week post transplantation, more B cells were generated from CLPs than LMPP-s and LMPP+s although the total numbers of B cells were very low and less than 0.2% of those at the 3 weeks time point (Figure A-2C). Significantly ($p<0.05$) more spleen NK cells ($TCR^{-}CD19^{-}$ $NK1.1^{+}$) were generated from LMPP+s than CLPs (Figure 3C). In addition to those lymphocytes, small numbers of myeloid cells ($CD11b^{+}TCR^{-}B220^{-}CD19^{-}NK1.1^{-}$) were also generated in the spleen from LMPP-s and LMPP+s but not CLPs (Figure 3D). The myeloid lineage potentials of LMPP-s and LMPP+s were also compared by granulocyte/monocyte (G/M) colony formation in vitro. More than 30% of the plated LMPP-s and less than 15% of plated LMPP+s formed G/M-colonies in vitro (Figure A-2D).

We also detected donor-derived ILC2s in the lung of the recipient mice 3 weeks post transplantation. Very few (less than 100 cells per mouse) ILC2s were generated from CLPs whereas the number of LMPP+ derived ILC2s was significantly ($p<0.01$) higher (Figure 3E). To test the capacities of the progenitors to differentiate into ILC3s, we purified CLPs, LMPP-s and LMPP+s from the RORγt reporter allele (*Rorct* $^{GFP/+}$) mouse BM [78] and transplanted them into lethally irradiated B6.Ly5SJL mice. Donor-derived ILC3s were detected in the small intestine of the recipients as $CD45.2^{+}CD45.1^{-}Lin^{-}CD127^{+}GFP^{+}$ cells. At 3 weeks after the transplantation, only small numbers (less than 50 cells per mouse) of ILC3s were detected. Nevertheless, significantly more ILC3s were generated from LMPP+s than CLPs or LMPP-s (Figure 3F). Thus, all three progenitor populations were capable of differentiating into T, B, NK, ILC2 and ILC3 cells. However, they were significantly different from each other in the numbers of progenies they generated in the transplanted mice. LMPP+s were potent progenitors for all the lymphoid lineages tested, particularly T cells, NK cells, ILC2s and ILC3s. In contrast, much smaller numbers of lymphocytes were

generated from CLPs, with the exception of B cells.

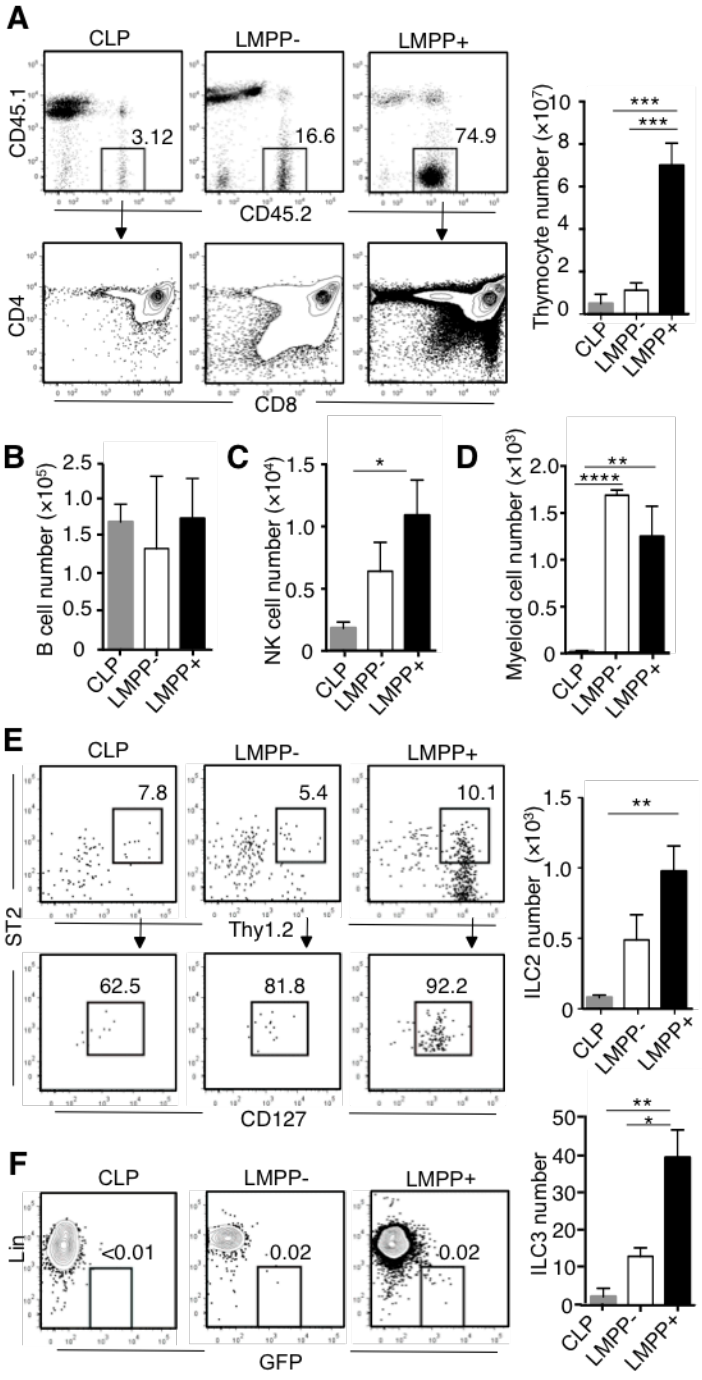

Figure 3 LMPP+ are more efficient than LMPP-s and CLPs at generating T cells and ILCs.

(A) CLPs, LMPP-s, or LMPP+s were purified by cell sorting from C57BL/6 mice ($CD45.2^+$),

and 2,000 cells of each population were intravenously injected with 10^5 helper BM cells from NSG mice ($CD45.1^+$) into lethally irradiated congenic $B6.Ly5^{SJL}$ recipients ($CD45.1^+$). Three weeks post-transplantation, the cells in the thymuses of the recipient mice were analyzed by flow cytometry. Live (PI^-) donor-derived cells ($CD45.1^-$ $CD45.2^+$) were gated (top row) and the expression of CD4 and CD8 were plotted (bottom row). The bar graph shows the absolute numbers of donor-derived cells in the thymus of the recipient mice.

(B) Absolute numbers of donor-derived B cells ($TCR\alpha\beta^-TCR\gamma\delta^-NK1.1^-CD19^+B220^+$) in the spleen of the transplanted mice 3 weeks post-transplantation were calculated from the frequency of donor-derived B cells as determined by flow cytometry and the total numbers of spleen cells.

(C) Absolute numbers of donor-derived NK cells ($TCR\alpha\beta^-TCR\gamma\delta^-CD19^-NK1.1^+$) in the spleen of the transplanted mice 3 weeks post-transplantation were calculate as in B.

(D) Absolute numbers of donor-derived myeloid cells ($TCR\alpha\beta^-TCR\gamma\delta^-CD19^-NK1.1^-$ $CD11b^+$) in the spleen of the transplanted mice one week post-transplantation were calculated as in B.

(E) The lungs of the recipient mice were analyzed 3 weeks post-transplantation for donor-derived ILC2s by flow cytometry. Live (PI^-) $CD45.1^-CD45.2^+Lin^-$ cells were first gated and ILC2s were gated by $Thy1^+ST2^+$ (top) and $CD127^+$ (bottom). The bar graph (right) shows the absolute number of donor-derived ILC2s in the lungs of transplanted mice.

(F) The intestines of the recipient mice were analyzed 4 weeks post-transplantation for donor-derived ILC3s. Donor-derived live $CD45.1^-$ $CD45.2^+$ cells were further gated for Lin^-GFP^+ (ILC3s). The bar graph (right) shows the absolute numbers of donor-derived ILC3s cells in the intestines of transplanted mice.

The absolute number of donor-derived cells in tissues of recipient mice was calculated by multiplying cell frequency by the total number of cells. Mean ± S.E.M., * = $p \leq 0.05$, ** = $p \leq 0.01$ and ***= $p \leq 0.001$ (two-tailed Student's t-test). Data are representative of at least three independent experiments with 4 mice in each condition. See also Figure A-2.

ß3.3 LMPP+s differentiate into CLP and CHILPs

To investigate the relationship between LMPP-s, LMPP+s and CLPs, we transplanted them into irradiated recipients and analyzed donor-derived cells in the BM one week later. LMPP-s differentiated into both $cKit^{hi}$ and $cKit^{lo}$ Lin^- cells expressing CD127 and Flt3, resembling LMPP+s and CLPs. In contrast, LMPP+s differentiated into $Lin^-Sca\text{-}1^{lo}cKit^{lo}Flt3^{hi}CD127^+$ CLPs but not LMPP-s (Figure 4A). These data suggest that LMPP-s are the most immature

whereas LMPP+s are intermediate between LMPP-s and CLPs, and CLPs are the most differentiated lymphoid progenitors.

Recently, common progenitors for all helper-like ILCs or CHILPs in the adult mouse BM have been identified by the expression of the transcription factor Id2 [10], [45]. As the above results showed that LMPP+s are more efficient than CLPs in differentiating into ILC2s, we compared their potential to give rise to CHILPs. We purified LMPP+s and CLPs from the Id2 reporter *Id2*$^{GFP/+}$ mouse BM, transplanted them into irradiated B6.Ly5SJL mice and analyzed BM cells for donor-derived CHILPs ($CD45.2^+CD45.1^-Lin^-Thy1.2^+CD25^-CD127^+ST2^-GFP^+$). Significantly ($p<0.05$) more CHILPs were generated from LMPP+s than CLPs one week post-transplantation although the numbers of donor-derived CHILPs were low (Figure 4B).

To investigate the differentiation of LMPPs and CLPs into the innate lymphocyte lineages in steady state, we analyzed the RAG-1Cre/Rosa26 tandem dimer red fluorescent protein (tdRFP) mice (Rag-1/Red), in which RAG-1 expression during lymphocyte development results in irreversible expression of tdRFP [79]. LMPP-s were almost all negative for RAG/Red whereas ~25% of LMPP+s and more than 65% of CLPs were RAG/Red$^+$ (Figure 4C). As expected, almost all T cells and a very small fraction of myeloid cells expressed RAG/Red. Only about 20% of splenic NK cells were RAG/Red+. The low percentages of RAG/Red$^+$ NK cells suggest that the majority of NK cells may develop from LMPP-s or LMPP+s while only a minor fraction, if any, may develop from CLPs. About ~33% of BM ILC2Ps and 40% of lung ILC2s were RAG/Red+. These results suggest that the majority of BM ILC2Ps may directly develop from LMPP+s with minor CLP contribution. The small but significant ($p<0.05$) difference in the percentages of RAG/Red$^+$ cells between

lung ILC2s and BM ILC2Ps also suggests that not all lung ILC2s develop from BM ILC2Ps and there might be minor contributions from CLPs to lung ILC2s. To further investigate if the development of ILC2s could bypass the CLP stage of development, we analyzed Flt3-ligand deficient (*Flt3l*$^{-/-}$) mice. In agreement with previous studies (Sitnicka et al., 2002; Sitnicka et al., 2007), our results showed that Flt3 ligand is necessary for the efficient generation of both LMPPs and CLPs. However, LMPP-s were less severely reduced than CLPs and LMPP+s in *Flt3l*$^{-/-}$ mice (Figure A-3A). ILC2Ps were also severely reduced, and yet near normal numbers of ILC2s were found in the lungs of *Flt3l*$^{-/-}$ mice (Figure A-3B-C). These results imply that the ILC2 development does not strictly require CLPs or LMPP+s as an intermediate stage and these cells can directly develop from the more immature LMPP-s. The results also suggest that some lung ILC2s may develop directly from LMPP-s bypassing the BM ILC2P stage. This is in contrast to the classically defined linear model of lymphopoiesis, which describes one pathway to lymphocyte development through CLPs.

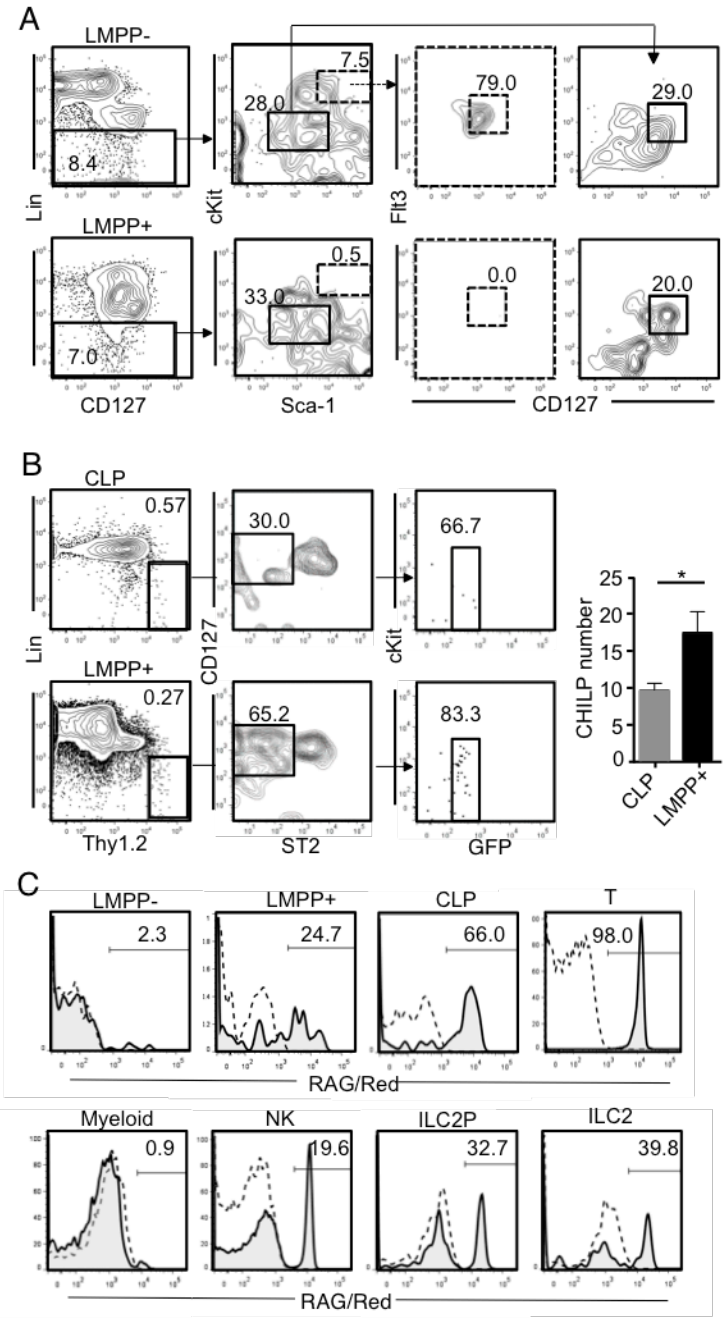

Figure 4 LMPP+s are the upstream lymphoid progenitors to both CLPs and CHILPs.

(A) Lethally irradiated B6.Ly5^{SJL} mice received intravenous injection of 2,000 cells each of purified LMPP-s (top row) or LMPP+s (bottom row) from adult B6 mice BM, along with 10^5 helper BM cells from NSG mice, and the BM were analyzed 1 week post-transplantation. Live donor-derived ($PI^-CD45.2^+$) leukocytes were gated for Lin^- (left), and further gated for the LMPP (dashed line) and CLP (solid line) populations and analyzed for Flt3 and CD127 expression.

(B) Lethally irradiated B6.Ly5^{SJL} mice received intravenously injection of 2,000 cells each of

LMPP+s and CLPs from BM of $Id2^{Gfp/+}$ mice, along with 10^5 BM cells from NSG mice. The BM of the recipient mice was analyzed 1 week post-transplantation. The donor-derived ($CD45.1^-CD45.2^+$) cells were gated for Lin^-Thy1^+ and further gated for $CD127^+ST2^-$, and CHILPs were identified by the expression of Id2 (GFP). The bar graph shows the absolute numbers of donor-derived CHILPs.

(C) Lethally irradiated $B6.Ly5^{SJL}$ mice received 10^6 BM cells from Rag-1/Red mice or normal B6 mice (control). Eight weeks later, cells in the BM, spleen and lung of the recipient mice were analyzed by flow cytometry. Donor-derived ($CD45.2^+$) $LMPP^-$, $LMPP^+$, CLPs and ILC2Ps were gated as in Figure 2A. T cells, myeloid cells and NK cells in the spleen and ILC2s in the lung were gated as in Figure 3. Histograms show the expression of tdRFP in indicated populations from Rag-1/Red mice (shaded) and from control mice (dashed line).

The numbers indicate the percentages of cells in the gates. The data are representative of 3 experiments. See also Figure A-3.

3.4 LMPPs are the predominant neonatal lymphoid progenitors when ILC2 and T cell development is highly active

To further study the development of lymphocytes, we analyzed progenitors in the perinatal period when lymphocyte development is very active. As ILC2Ps and ILC2s were undetectable in the fetal liver and lung (data not shown), we analyzed neonatal tissues. In 1 day old mice, there were very few $CD45^+Lin^-$ cells and almost undetectable numbers of CLPs and LMPP+s in the BM. Instead, lymphoid progenitors present in the 1 day old spleen were predominantly LMPP+ while CLPs were barely detectable (Figure 5A). Similarly, ILC2Ps were undetectable in the BM of 1 day old mice, but abundant in the spleen at this time point. In 10 days old mice, lymphoid progenitors were found in both the BM and spleen. Only ~300 ILC2s in the lung of 1 day old mice and more than 1,500 ILC2s in 5 day old mice were detected. The number of lung ILC2s rapidly increased to more than 15,000 in 10 day old mice, whereas there were around 5,000 in the adult lungs (Figure 5B). Thymopoiesis is also known to be very active in the early postnatal life [80]. In agreement with the published findings [81], the frequencies of ETPs, defined as $Lin^{lo}cKit^{hi}CD25^-$ thymocytes, in the thymus were much higher in neonatal (3 day old) compared to adult mice (Figure A-4A).

The total number of ETPs peaked in 10 day old mice to three times those of adults, 3 and 5 day olds (Figure 5C). Therefore, there appeared to be a wave of rapid expansion of both lung ILC2s and thymus ETPs between 5 days and 10 days after birth.

To elucidate the pathways of neonatal ILC2 and ETP development, we then analyzed for LMPP+s, CLPs and CHILPs at different time points after birth. Neonatal BM contained small numbers of LMPP+s but barely detectable CLPs until 5 days after birth. In contrast, LMPP+s and CLPs were readily detectable in neonatal spleens as young as 1 day after birth. Unlike in adult BM, LMPP+s were much more abundant than CLPs in neonatal spleens (Figure 5D and Figure A-4B). CHILPs and ILC2Ps were also readily detected in the spleen of neonatal mice (Figure 5E). These observations suggested that LMPP+s, but not CLPs, in the spleen are the major progenitors for ETPs, ILC2s and CHILPs in neonatal (up to 5 days old) mice. The site of lymphopoiesis slowly shifts from the spleen to BM but CLPs remain a minor population until 10 days after birth.

We also analyzed lymphoid progenitors in the peripheral blood of neonatal mice. Almost five times more LMPP+s than CLPs were detected in the neonatal blood (Figure A-4C). Interestingly, high frequencies of ILC2-like cells were also found in the neonatal blood. CHILPs or other lymphoid progenitors were not detected in neonatal lungs, and transplantation of different subsets of neonatal lung lymphocytes failed to generate donor-derived ILC2s or other lymphocytes in the recipients' lungs (data not shown). Thus, in neonatal mice ILC2s seem to develop mainly in the spleen and migrate through the blood to the lung.

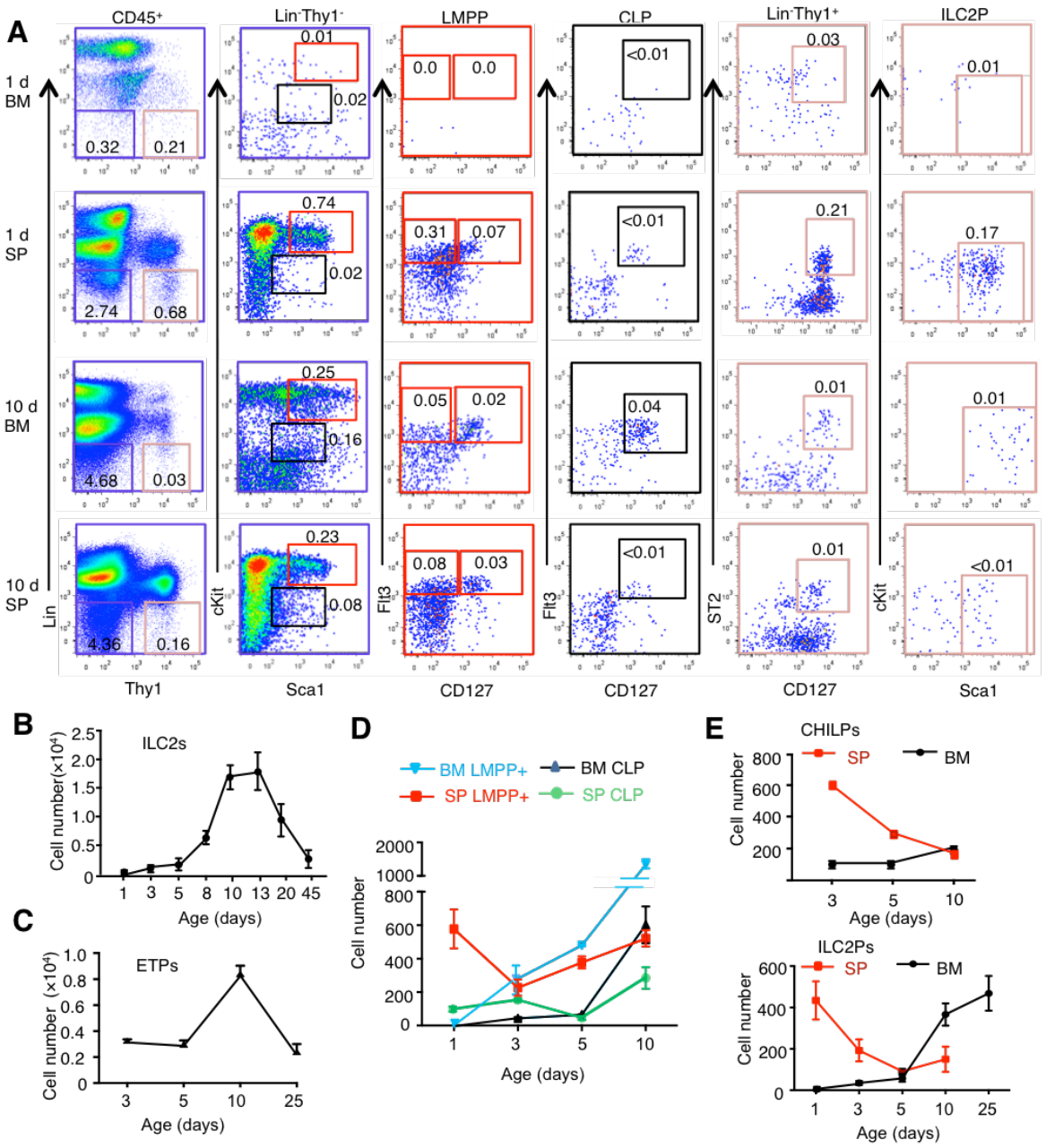

Figure 5 LMPP+s are prominent lymphoid progenitors in neonatal BM and spleen, when ILC2 and T cell development is highly active.

(A) The BMs and spleens of 1 and 10 day old neonatal C57BL/6 mice were analyzed by flow cytometry. Live (PI^-) $CD45^+$ cells were first gated for Lin^- $Thy1^-$ (blue gate) and Lin^-Thy1^+ (brown gate) cells. Lin^-Thy1^- were then gated for $cKit^{hi}Sca\text{-}1^+$ (red gate) and $cKit^{lo}Sca\text{-}1^{lo}$ (black gate). $Flt3^{hi}CD127^-$LMPP-s, $Flt3^{hi}CD127^+$LMPP+s, and CLPs were identified by the expression of Flt3 and CD127. ILC2Ps among Lin^-Thy1^+ cells were identified by $ST2^+CD127^+cKit^{lo/-}Sca1^{hi}$. Numbers in plots indicate mean percentages of gated cells among live $CD45^+$ cells.

(B) Absolute numbers of lung ILC2s were calculated and compared at different neonatal and adult time points.

(C) Absolute numbers of ETPs were calculated and compared at different neonatal and adult time points.

(D) Absolute numbers of LMPP+s and CLPs from spleen and BM were calculated and compared at different neonatal time points.

(E) Absolute numbers of CHILPs defined as $CD45.2^{+}CD45.1^{-}Lin^{-}$ $Thy1.2^{+}$ $CD25^{-}CD127^{+}$ $ST2^{-}GFP^{+}$ (top) and ILC2Ps (bottom) from spleen and BM of $Id2^{Gfp/+}$ mice were calculated and compared at different neonatal time points.

The data in A are representative of 4 independent analyses. The data in B – D are the mean of 4 independent analyses and error bars show the SEM. See also Figure A-4.

3.5 Neonatal LMPP+s are potent T cell and ILC2 progenitors

To examine the lymphoid differentiation capacities of neonatal LMPP+s and CLPs, they were purified from 3-6 days old mouse spleens, and transplanted into irradiated congenic $B6.Ly5^{SJL}$ recipients. In comparison with CLPs, LMPP+s showed robust thymic engraftment and generated significantly (<0.05) more T cells two weeks post-transplantation (Figure 6A). We then compared these neonatal spleen lymphoid progenitors and CHILPs for their ILC2 potential. In addition to producing T cells, neonatal LMPP+s efficiently gave rise to ILC2s in the lung (Figure 6B). Comparable numbers of ILC2s were generated from CHILPs. These results suggest that LMPP+s are the main source of T cells and ILC2s in the neonatal stage.

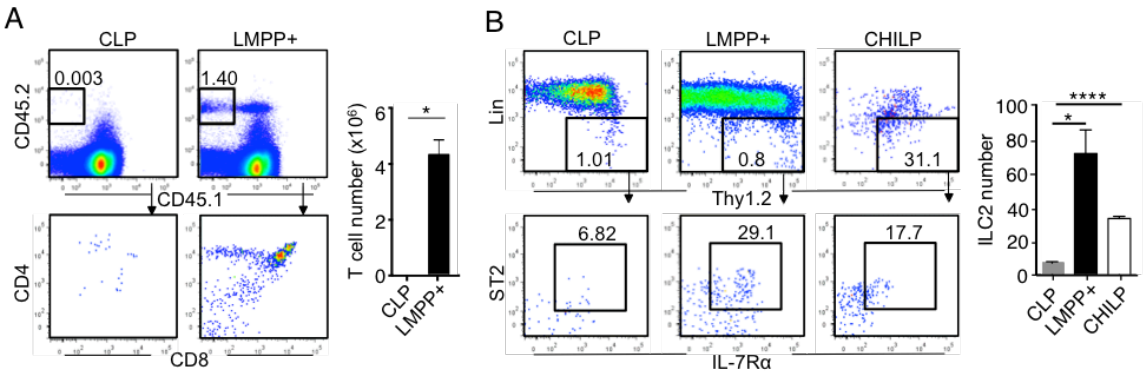

Figure 6 Neonatal LMPP+s are more efficient than CLPs at generating T cells and ILC2s.

(A) CLPs or LMPP+s were purified by cell sorting from pooled spleen cells from 3-6 day old C57BL/6 mice and 1,125 cells each, along with 10^5 BM cells from NSG mice, were intravenously injected into lethally irradiated congenic B6.Ly5^{SJL} recipients. The thymuses of the recipient mice were analyzed 2 weeks post-transplantation. Donor-derived (CD45.1$^-$ D45.2$^+$) cells were gated (top) and analyzed for CD4 and CD8 expression. The bar graph shows the absolute numbers of donor-derived cells in the recipients' thymus. Numbers in plots indicate the mean percentages of the cells in the gates.

(B) The lungs of the recipient mice were analyzed 2 weeks post-transplantation. Live (eF780$^-$) donor-derived (CD45.1$^-$CD45.2$^+$) cells were gated for Lin$^-$ Thy1$^+$ (top row) and ILC2s were further gated by CD127$^+$ST2$^+$ (bottom row). The bar graph shows the absolute numbers of donor-derived ILC2s (Mean ± S.E.M) in the lungs of transplanted mice.

* = $p \leq 0.05$, ** = $p \leq 0.01$ and ***= $p \leq 0.001$ (two-tailed Student's t-test). Data are representative of at least two independent experiments with 4 mice in each condition.

Chapter 4 Discussion

In the conventional scheme of lymphocyte differentiation, all lymphocytes develop from CLPs that develop from HSCs in a single linear pathway of HSC → LMPP → CLP → committed progenitors (pre-T, Pre-B, NKP, CHILP) → mature T, B, NK and ILCs. However, our current study suggests that many T cells and ILCs likely develop from LMPP+s bypassing the CLP stage, particularly in neonatal mice. LMPP+s significantly differ from the conventional LMPPs, termed LMPP-s in our study, in the capacities to differentiate into lymphocytes and myeloid cells as well as in their gene expression profiles. In transplantation assays, the LMPP+s are much more efficient than the LMPP-s in thymic engraftment and in differentiating into ILCs whereas LMPP+s are less efficient than LMPP-s in forming myeloid colonies in vitro. The genes associated with lymphoid development including *Il7r* and *Rag2* are more highly expressed in LMPP+s than LMPP-s, however, the expression of myeloid-associated genes including *Clec9a* and *Irak3* is lower in LMPP+s than LMPP-s. LMPP+s and CLPs are also very different from each other in their ability to differentiate into all lymphoid lineages examined with the exception of B cells. Our analyses of neonatal mice have also shown that CLPs are scarce compared to LMPP+s. In newborn mice (1 day old), the BM contains very few $CD45^+$ cells whereas LMPP+s, CHILPs, ILC2Ps are readily detected in the spleen and a small number (less than 500) of ILC2s are found in the lung. Thus, in the first 3 days after birth, lymphopoiesis seems to take place in the spleen rather than BM. Although LMPP+s become detectable in the BM 3 days after birth, the numbers of CLPs remain much lower than LMPP+s in neonatal spleen and BM up to 10 days after birth when T cell development is very active and ETPs are found in large numbers in the thymus. ILC2 development is also very active for the first 10 days after birth as the number of ILC2s in the lung increases significantly. Transplantation experiments also confirmed that neonatal

CLPs have very low capacity to differentiate into T cells and ILC2s. Therefore, most neonatal T cells and ILC2s likely develop from LMPP+s through a CLP-independent pathway.

LMPP+s and CLPs in our study likely overlap with All-Lymphoid Progenitors (ALPs) and B cell-biased Lymphoid Progenitors (BLPs), respectively, described as subsets of CLPs by the Weissman group [82]. Whereas CLPs are defined, as clonogenic common lymphoid progenitors with equal potential to give rise to all lymphocytes and no myeloid potential [32], [33], LMPP+s (and ALPs) have myeloid potentials as they differentiate into myeloid cells in transplantation assays as well as in vitro colony assays. Therefore, LMPP+s do not fit the classical definition of CLPs. Instead, LMPP+s are more similar to ELPs reported by the Kincade group [36]. ELPs were defined by the expression of RAG reporter gene. Although, ELPs and LMPP+s express the RAG reporter gene and have high capacities to differentiate into T cells in transplantation assays, ELPs were reported to be $CD127^{-}$. As a small subset of LMPP-s also expresses the RAG reporter gene, the exact relationship between ELPs and LMPP+s is still unclear. LMPP+s might be the adult counterparts of the recently described fetal lympho-myeloid progenitors [31]. These fetal progenitors are Lin^{-} $cKit^{hi}Flt3^{+}CD127^{+}Rag1^{+}$ cells and phenotypically resemble the LMPP+s in our study. They emerge at embryonic day (E) 9.5 in the yolk sac and E10.5 in the fetal liver. Interestingly, these cells co-expressed the key lymphoid and myeloid genes and showed a robust contribution to both myeloid and lymphoid lineages. The differentiation capacities of these fetal progenitors have been tested by in vitro cultures, but not transplantation assays, and their relationship with postnatal LMPP+s is currently unclear.

Although LMPP-s, LMPP+s and CLPs in adult mice are all capable of differentiating

into the lymphoid lineages including T, B, NK cells and ILCs in transplantation assays, LMPP+s are far more efficient than the other progenitors in their capacity to repopulate the thymus. In vitro differentiation cultures on OP9-DL1 stroma, demonstrated that all three progenitors efficiently differentiated into immature T cells. Thus, the highly efficient thymic engraftment of LMPP+s as compared to the other progenitors is likely due to their ability to migrate to the thymus. Consistent with this notion, our microarray analysis showed that the expression of the gene encoding the chemokine receptor CCR9, which is thought to be important for trafficking into the thymus [83], is significantly higher in LMPP+s than LMPP-s. Whereas LMPP-s are able to differentiate into LMPP+s in transplanted mice, they likely have to migrate into the BM first for the differentiation into LMPP+s. LMPP+s also seem to be the major T cell progenitors in steady state, not just in transplantation assays. They are more abundant than CLPs in neonatal mice when intrathymic T cell differentiation is most active. Furthermore, LMPP+s are readily detected in the peripheral blood of adult and neonatal mice, suggesting that LMPP+s emigrate from the adult BM and neonatal spleen, circulate and seed the thymus.

LMPP+s are also more efficient than CLPs in differentiating into innate lymphocytes including NK cells, ILC2s and ILC3s in transplantation assays. While the generation of more ILC2s in the lung [72] and ILC2Ps in the BM (Halim et al. 2012) from LMPPs than CLPs has previously been reported, our current transplantation assays have shown that LMPP+s are responsible for the efficient generation of the common ILC progenitors termed CHILPs and subsequent ILC2 and ILC3 development. The lineage tracking by RAG-Cre mediated RAG/Red expression also shows that the percentage of RAG/Red positive cells among BM ILC2Ps (~33%) is significantly ($p<0.001$) lower than those among CLPs (~65%) and slightly higher ($p<0.05$) than those among LMPP+s (~25%). Similarly, only about 20% of NK cells

in adult mouse lung and spleen express RAG/Red. Therefore, most ILC2s and NK cells seem to develop from LMPP+s before they differentiate into CLPs, whereas CLPs and LMPP-s also seem to directly contribute to the ILC2 and the NK cell lineages, respectively, to lower degrees. Although CLPs are inefficient in differentiating into lung ILC2s in transplantation assays, normal adult mouse BM contains five times more CLPs than LMPP+s. Therefore, CLPs likely contribute to the ILC2 lineage to some extent in adult mice as suggested by the lineage tracking analysis by RAG/Red. However, our results with *Flt3l*$^{-/-}$ mice, which have greatly reduced CLPs but near normal numbers of lung ILC2s, indicate that the development of ILC2s can bypass the CLP stage. Furthermore, in the neonatal period when T cells and ILC2s actively develop, CLPs comprise a very minor population and they have very low capacities to differentiate into T cells and ILC2s in transplantation assays. In contrast, LMPP+s are prominent in neonatal mice and they have high capacities to differentiate into T cells and ILC2s. These results further support the existence of CLP-independent developmental pathways for these cells.

Whereas our transplantation assays have shown the differentiation processes of LMPP- → LMPP+ → CLP, our data do not fit the conventional model of this linear single pathway of lymphocyte development. Instead, it is more likely that different lymphoid lineages start branching out at different stages as delineated in (Figure 7). The NK cell lineage seems to be the first to branch out from LMPP- and continues until the LMPP+ stage whereas the ILC lineage appears to start branching out mainly at the LMPP+ stage but continues until the CLP stage. Although all the progenitors have the capacities to differentiate into T cells and B cells, most B cells seem to develop from CLPs whereas most T cells likely develop from LMPP+s. CLPs retain the capacities to differentiate into all lymphoid lineages and seem to contribute to the T cell and the ILC2 lineages to some degree

in adult mice. In contrast, CLPs are scarce in neonatal mice when T and ILC2 development is very active, and their contribution is likely insignificant. Our model of non-linear differentiation of lymphocytes from multiple progenitors with varying probabilities clarifies confusions in the field. As the development of the immune system in the neonatal period is thought to impact immunity in later life, our results with neonatal lymphocyte development will also have important implications to our understanding of the development of the immune system.

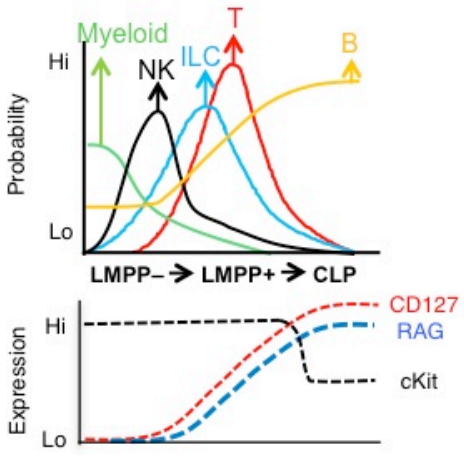

Figure 7 Schematic representation of non-linear model of lymphocyte development.

The histograms (top) depict the probability of LMPP-s, LMPP+s and CLPs differentiating into indicated cell lineages, whereas the bottom graph depicts the changes in the expression of CD127, RAG1 and cKit in these progenitors. LMPP-s have a low probability to differentiate into the myeloid lineage (green histogram) whereas the probability further declines as LMPP-s differentiate into LMPP+s and it is lost as they further differentiate into CLPs. The probability of differentiating into the NK cell lineage (black) is high in the LMPP- and LMPP+ stages and it declines to a very low level as they differentiate into CLPs. LMPP+s have high probabilities of differentiating into the ILC (blue) and T cell (red) lineages, whereas the probabilities of LMPP-s and CLPs differentiating into the ILC and T cell lineages are lower. In this model, LMPPs differentiate into multiple lymphoid lineages

without differentiating into CLPs. CLPs also have the capacities to differentiate into the ILC, T and NK cell lineages, but with only low probabilities. Thus, individual lymphoid lineages can develop from multiple progenitors with varying probabilities.

Chapter 5 General summary

5.1 General summary

It is thought that all lymphocytes develop from a single population of committed lymphoid progenitors in the BM termed CLPs. CLPs derive from the upstream lymphoid progenitors termed LMPPs that have the capacities to differentiate into myeloid and multiple lymphoid lineages. Thus, the classical model of lymphocyte development implicates a single linear differentiation pathway HSC → LMPP → CLP → lineage committed progenitors (pre-T, pre-B etc.) → mature T, B and NK cells. However, CLPs are not efficient T cell progenitors and the identity of T cell progenitors in the BM has been debated.

In addition to T, B and NK cells, the lymphocyte population now includes ILCs, which are divided into three groups: ILC1, ILC2 and ILC3. Common progenitors for all ILCs, termed CHILPs or ILCPs, have also been identified. As the ILCPs can be generated from CLPs in vitro, the ILC lineages are thought to develop from CLPs. However, we have previously found that LMPPs are much more efficient than CLPs in the generation of BM ILC2 progenitors and mature lung ILC2s in transplantation assays (Halim et al. 2012). Our results are consistent with a report from the Bhandoola group. These results suggested that a CLP-independent pathway of ILC2 development might exist. To clarify the contradictory data, we now have carried out detailed analyses of lymphoid progenitors by transplantation assays and lineage tracking by RAG-1 expression. We have also analyzed lymphocyte development in neonatal mice.

The scientific questions being asked by our current study are:

- Do all lymphocytes develop from CLPs?
- Is there a CLP-independent developmental pathway for innate lymphocytes?

- From which lymphoid progenitors do neonatal ILC2s and T cells develop?

The principal new findings are:

- ✓ The LMPP population can be divided into two subsets based on the expression of CD127 (IL-7Rα). The $CD127^-$ subset (termed $LMPP^-$) and the $CD127^+$ subset (termed LMPP+) significantly differ in their capacities to generate T cells in transplantation assays as well as gene expression profiles. LMPP-s differentiate into LMPP+s and CLPs whereas LMPP+ differentiate into CLPs but not LMPP- in transplanted mouse BM.
- ✓ LMPP+s are much more efficient than CLPs in generating ILCs, CHILPs, NK and T cells in transplantation assays.
- ✓ LMPP+s are detected in the peripheral blood of adult and neonatal mice whereas CLPs are very rare.
- ✓ Lineage tracking by RAG-1 reporter expression shows that most ILC2s and NK cells develop from the LMPP subsets.
- ✓ Flt3L-knockout mice have severely reduced CLPs, LMPP+s and ILC2 progenitors in the BM and yet near normal numbers of ILC2s in the lung.
- ✓ In the first 10 days after birth, CLPs are rare and, unlike prominent neonatal LMPP+s, incapable of differentiating into ILC2s and T cells while their development is highly active.

5.2 Significance

The work in this thesis challenges the conventional scheme of lymphocyte differentiation and the idea of "common lymphoid progenitors" as an obligatory stage for the development of all lymphocytes. We propose a model for the differentiation of lymphocytes within which different lymphoid lineages branch out from multiple progenitors with varying probabilities

rather than all differentiating from CLPs. In this model LMPPs have very high probabilities to differentiate into NK, ILC and T lineages directly, without differentiating into CLPs. The data provided here significantly contribute to our better understanding of lymphopoiesis.

BM transplantations are often preceded by lethal irradiation. Following BM transplantation, peripheral T cell pool, which is important in patients defense against infections, mainly recovers through thymus dependent maturation of the transplanted progenitor cells. Due to the radiosensitivity of thymic epithelial cells, irradiation can limit the chemokine signaling on thymic endothelium and slow down thymus settling of transplanted progenitors and T cell reconstitution. It is important to know which lymphoid progenitors are the most efficient progenitors in settling in and reconstituting the thymus in this condition. However, significant differences might also exist between mice and the human lymphopoiesis and further study is required to reveal the existence of such progenitors in human BM.

Technical limitations of my work include the requirement for transplantation of lymphoid progenitors into lethally irradiated hosts to investigate their potential. Irradiation can severely disrupt the steady state hematopoiesis, and only cells that are able to colonize a niche will be able to generate detectable progeny. Additionally considering the stress that transplanted cells tolerate during their isolation as well as engraftment, it is possible that the differentiation capacities of progenitors in transplantation assays may not mimic their differentiation in normal steady state lymphopoiesis. Therefore, we have extended our studies to include the lineage tracking mouse model of RAG-Cre mediated RAG/Red expression. The model provides a valuable index of the history of RAG-1 expression during lymphocyte development and relatedness of mature lymphoid cells to their upstream

lymphoid progenitors. In this model, most ILC2s and NK cells seem to be more related to LMPP+s than CLPs by means of their RAG/Red expression.

Innate lymphocyte development in the neonatal period has not been extensively studied. My thesis study has shown that neonatal and adult mice significantly differ in lymphoid progenitors and the development of T cells and ILC2s is highly active in the neonatal period. As the development of immune system early in life is thought to impact adult immunity, this study has important implications to our understanding of the development of the immune system.

5.3 Future directions

5.3.1 Affymetrix microarray analysis of CLPs

We have compared LMPP-s and LMPP+s by Affymetrix microarray analysis and realized that the expression of the lymphoid lineage-associated genes is generally higher in LMPP+s than LMPP-s. LMPP+s also expressed the myeloid cell lineage-associated genes albeit in lower levels than LMPP-s. We would like to carry out the same analysis for CLPs and compare the gene expression profile of these cells with the ones from LMPP+s. This comparison would allow us to investigate how exactly the expression of the lymphoid lineage associated genes are different between LMPP+s and CLPs. The differential expression of myeloid genes by these two populations may also explain the observed differences in their in vitro and in vivo myeloid potential.

5.3.2 Assessment of clonality

In order to track the clonality of cells within the LMPP+s we would like to do barcoding experiments. In cellular barcoding, integration of viral vectors with random sequence tags or barcodes will introduce a unique and heritable mark into the host cell genome. A sequencing-

based detection system can then be used to detect the clonal progeny of each cell (Gerrits et al., 2010). The barcoding experiments would enable us to assess the existence of different clones and heterogeneity within the LMPP+s. We will be able to investigate the existence of CILP as the ILC-restricted progenitor to both cytotoxic and cytokine producing ILCs among the LMPP+s. Also we will be able to investigate if the myeloid contribution of the LMPP+s is from the same clones that give rise to lymphocytes. Although the caveats associated with barcoding experiments such as in vitro manipulation of the progenitors as well as the required transplantation step in most of these experiments can compromise LMPP+s potentials in comparison with their steady state hematopoiesis.

5.3.3 Further analysis of the neonatal development of lymphocytes

Lymphocyte development is very active during neonatal period. We have carried out phenotypic analysis of the lymphoid progenitors in different hematopoietic tissues at this stage. LMPPs including both LMPP-s and LMPP+s were the predominant lymphoid progenitors during neonatal stage. We have compared the differentiation capacities of spleen LMPP+s with CLPs and realized that LMPP+s are much more efficient progenitors for ILC2s and T cells like their adult counterparts. We would like to extend our observations including LMPP-s in our comparison. We would also like to compare the differentiation potential of the same progenitors from neonatal spleen and BM to investigate if there is any difference regarding their lineage potential.

We would like to investigate the neonatal development of B cells. CLPs are the main B cell progenitors in adults. In the neonatal period CLPs are scarce and there are intrinsic differences between neonatal and adult B cells. I would like to investigate if different lymphoid progenitors would contribute to neonatal and adult stage B cell development.

5.3.4 ILC2s similarities with T cells

ILC2s phenotypically resemble double-negative 3 (DN3) thymocytes that are committed to the T cell fate. DN1 and DN2 thymic progenitors can give rise to ILC2s *in vitro*. However, ILC2s can develop normally in nude mice, indicating that the thymus is not required for their development (Wong et al., 2012). Nevertheless, the same transcription factors regulate the development of ILCs and T cells, and there are many similarities in their gene expression profiles. ILC2s express *Hes1*, *Dtx1* and *Lat* that are highly expressed by DN3 thymocytes. Also, ILC2s and DN3 thymocytes express comparable levels of *Il7ra*, *Il17rb* and *Il2ra* encoding cytokine receptors (Yang et al., 2013). While my study has shown that T cells and ILCs share the same progenitors (LMPP+s), it remains an open question whether T cells and ILCs have a close developmental relationship.

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Appendices

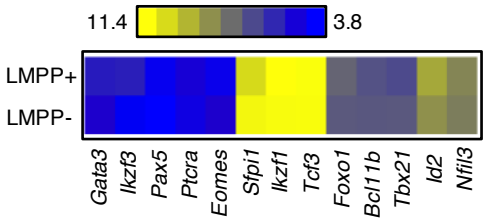

Figure A-1 Transcription factors required for early lymphoid lineage development.

Expression of the genes associated with lymphocyte developmental programs in LMPP+s was compared to those of LMPP-s by Affymetrix microarray analysis. The heat map shows the mean values from triplicate analyses.

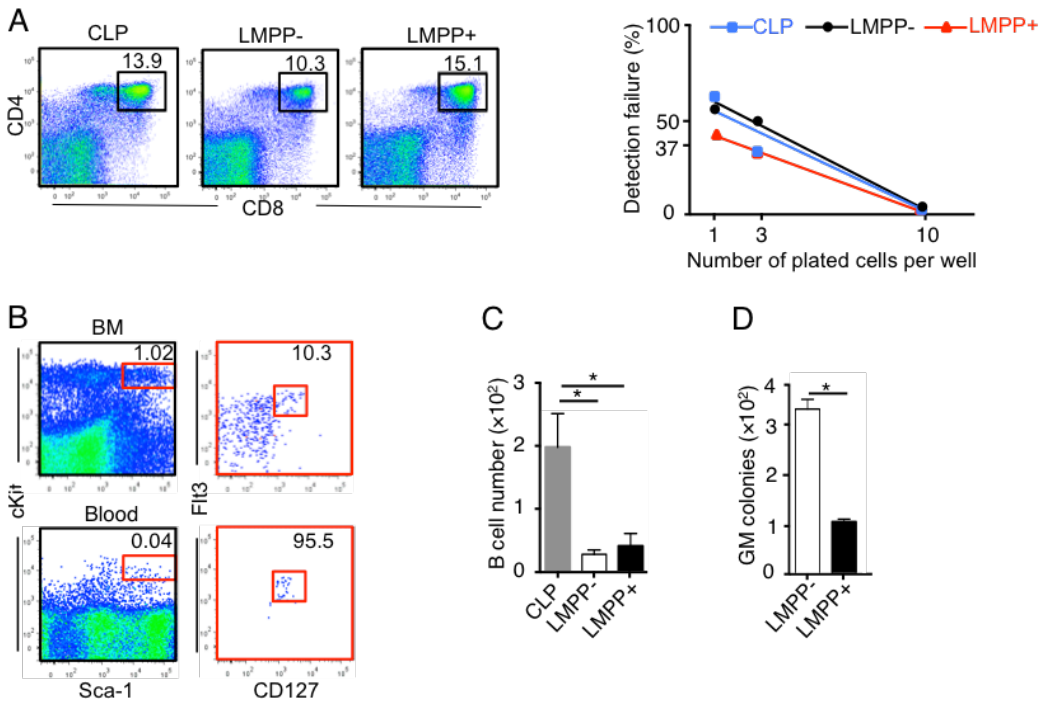

Figure A-2 BM LMPP+s are potent progenitors for T cells and ILCs.

(A) The T cell differentiation potential of purified CLPs, LMPP-s, or LMPP+s was assessed by limiting dilution cultures on OP9-DL1. After 21 days in cultures, wells with growing cells were scored and the cells in every 6 wells were pooled and analyzed by flow cytometry for the expression of CD4 and CD8. The frequencies of T cell progenitors were 1/2 for CLPs, 1/3 for LMPP-s and 1/2 for LMPP+s. The differences between the slopes are not significant (P=0.5261). Numbers in plots indicate mean percentages of gated cells among live $CD45^+$ cells.

(B) Phenotype of adult blood $Lin^-cKit^{hi}Sca\text{-}1^+$. The blood of 20 adult (6-8 weeks old) mice were pooled and red blood cells were lysed. Blood leukocytes and age matched BM cells were analyzed by flow cytometry. Live (PI^-) $CD45^+Lin^-$ were gated and $cKit^{hi}Sca1^+$ cells (red box) were analyzed for Flt3 and CD127 expression. The numbers in the plots show the percentages of the cells in the gates.

(C) Absolute numbers of B cells ($TCR\alpha\beta^-TCR\gamma\delta^-NK1.1^-CD19^+B220^+$) derived from CLPs, LMPP-s and LMPP+s in the spleen of the transplanted mice one week post-transplantation were calculated.

(D) LMPP-s and LMPP+s were purified from adult BM and 1,000 cells per plate were cultured in Iscove's MDM-based methylcellulose cultures for a monocyte/granulocyte colony forming assay for 14 days. Data are the mean of triplicate cultures in one of 2 independent experiments, both showing similar results.

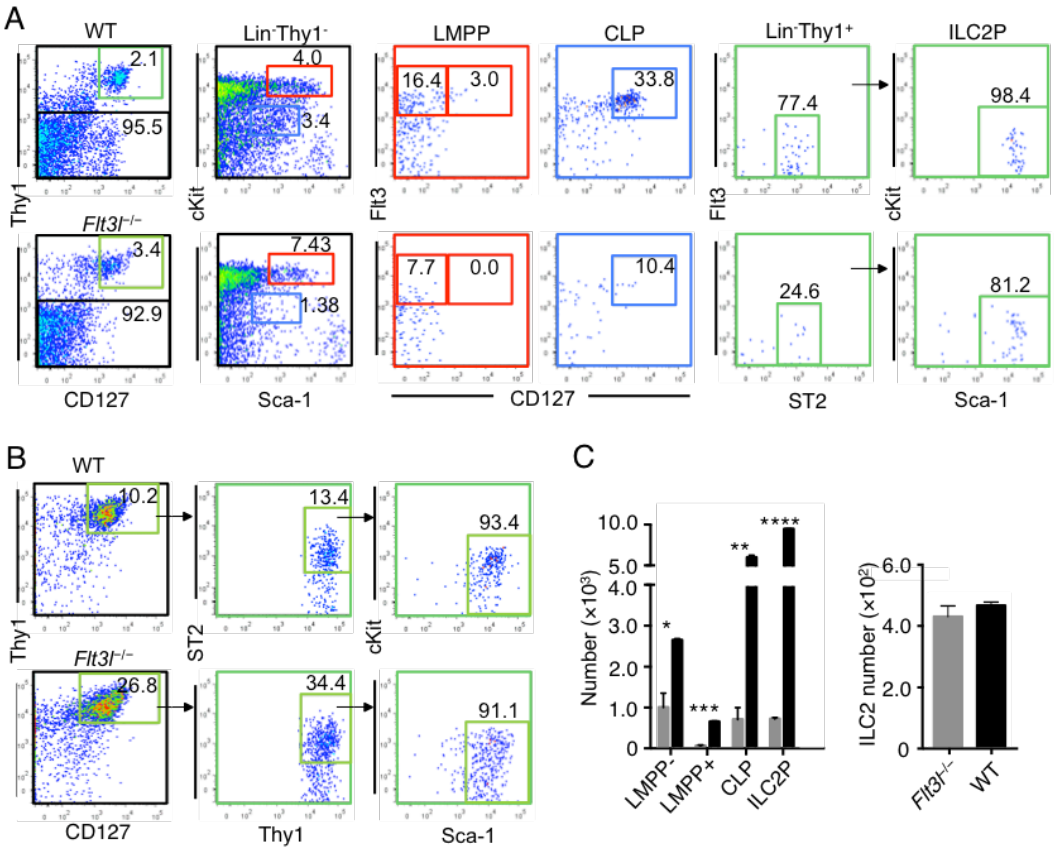

Figure A-3 Normal numbers of ILC2s were found in the lungs of *Flt3l*$^{-/-}$ mice.

(A) Flow cytometry analysis of lymphoid progenitors in the BM of C57BL/6 and *Flt3l*$^{-/-}$ mice. Live (PI$^-$) CD45$^+$Lin$^-$ cells were first gated for Thy1$^-$ (black box) and Thy1$^+$CD127$^+$ (green box) cells. Thy1$^-$ cells were then gated for cKit^{hi}Sca-1$^+$ (red gate) and cKit^{lo}Sca-1^{lo} (blue gate), and cKit^{hi}Sca-1$^+$ cells were further gated for Flt3^{hi}CD127$^-$ LMPPs (LMPP-s) and Flt3^{hi}CD127$^+$ (LMPP+s) (red boxes) while cKit^{lo}Sca-1^{lo} cells were gated for Flt3^{hi}CD127$^+$ (CLPs) (blue box). Thy1$^+$CD127$^+$ were gated for Flt3$^-$ST2$^+$cKit$^{lo/-}$Sca1^{hi} ILC2Ps (green gate). Numbers in plots indicate mean percentages of the cells in the indicated gates.

(B) Flow cytometry analysis of ILC2s in the C57BL/6 and *Flt3l*$^{-/-}$ mouse lung. Live (PI$^-$) CD45$^+$Lin$^-$ cells were gated for CD90$^+$CD127$^+$, ST2$^+$ and cKit$^{lo/-}$Sca1^{hi} ILC2s. Numbers in plots indicate mean percentages of the parent gates.

(C) Absolute numbers of BM lymphoid progenitors (left) and lung ILC2s (right) from C57BL/6 and *Flt3l*$^{-/-}$ mice were calculated.
Mean ± S.E.M., * = $p \leq 0.05$, ** = $p \leq 0.01$ and ***= $p \leq 0.001$ (two-tailed Student's t-test). Data are representative of at least three independent experiments with 4 mice each condition.

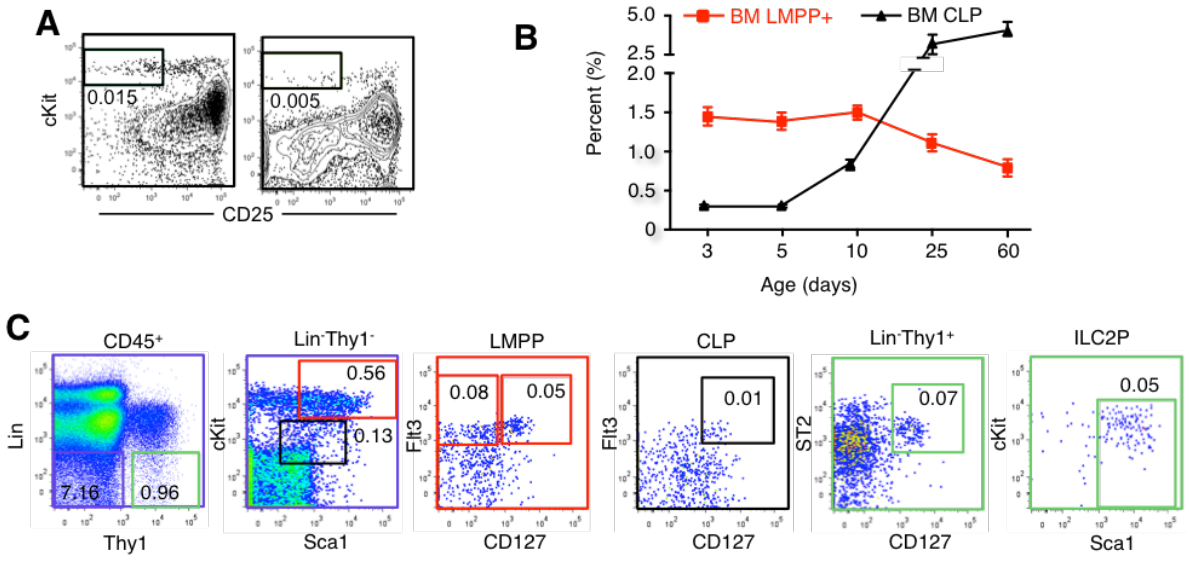

Figure A-4 LMPPs are the predominant neonatal lymphoid progenitors when ILC2 and T cell development is highly active.

(A) Flow cytometry analysis of ETPs in the thymus of 3 day old (left) and 45 day old (right) C57BL/6 mice. The plots were gated on live (PI^-) $CD45.2^+Lin^{lo}$ cells and further gated for $cKit^{hi}CD25^-$ ETPs. Numbers in plots indicate mean percentages of gated cells among live $CD45^+$ cells.

(B) The frequencies of BM LMPP+s and CLPs among $CD45^+$ cells at different ages were calculated and compared.

(C) Flow cytometry analysis of circulating lymphoid progenitors in the blood of neonatal mice. The blood from 16 neonatal (3 days old) mice was pooled and red cells were lysed. Live (PI^-) $CD45^+$ cells were gated for Lin^-CD90^- (blue gate) and Lin^-CD90^+ (green gate) cells. Lin^-CD90^- were then gated for $cKit^{hi}Sca\text{-}1^+$ (red gate) and $cKit^{lo}Sca\text{-}1^{lo}$ (black gate) while $cKit^{hi}Sca\text{-}1^+$ cells (red gate) were further gated for $Flt3^{hi}IL\text{-}7R\alpha^-$ LMPPs (LMPP-s) and $Flt3^{hi}CD127^+$ (LMPP+s). CLPs among $cKit^{lo}Sca\text{-}1^{lo}$ cells (black gate) were defined by $Flt3^{hi}CD127^+$. ILC2Ps among Lin^-CD90^+ were identified by $ST2^+CD127^+cKit^{lo/-}Sca1^{hi}$. Numbers in plots indicate the percentages of the gated cells among $CD45^+$ cells.

Table A-1.Defining mouse ILCs

	Lineage	Surface Markers		Transcription factors	Signature Cytokines
Cytotoxic ILCs	**NK cells**	Lin^-CD122^+ $NK1.1^+NKp46^+Ly49^+NKG2A^+$ $NKG2D^+$		Eomes, E4BP4 (NFIL3)	IFN-γ, TNF
Cytokine producing ILCs -	**ILC1**	$Lin^-IL7R\alpha^{+/-}Thy1^+NK1.1^+NKp46^+$		T-bet	IFN-γ, TNF
	ILC2	$Lin^-IL7R\alpha^+Thy1^+CD25^+ST2^{+/-}$ $Sca\text{-}1^+IL\text{-}17RB^+$		RORα, GATA-3, TCF-1	IL-5, IL-13
	ILC3	**LTi cells**	$Lin^-IL7R\alpha^+Thy1^+NKp46^-$ $cKit^{hi}CCR6^{hi}$	RORγt	LTs, IL-17 IL-22
		ILC3s	$LinIL7R\alpha^+Thy1^+$ $NKp46^+cKit^{int}CCR6^{lo/-}$	RORγt, AHR	IL-22

ILC, innate lymphoid cell; Lin, lineage markers (CD3, CD4, CD8, CD19, B220, GR1, CD11b, CD11c and TER119); $^-$IL7Rα, IL7 receptor subunit-α; NKp46, NK cell p46-related protein; NKG2A and Ly49 receptors, MHC-1 specific NK receptors; NKG2D, NK cell stimulatory receptor; IL, interleukin; CD25, IL2 receptor subunit-α; ST2, IL33 receptor; IL-17RB; Il17 receptor B; CCR6, c-c chemokine receptor type 6; IFN, interferon; TNF, tumor necrosis factor; LT, lymphotoxin.

Table A-2.Genes encoding the listed transcription factors

Genes	Transcription factors
Id2	Inhibitor of DNA-binding 2 (ID2)
Zbtb16	Promyelocytic leukemia zinc finger (PLZF)
Nfil3	Nuclear factor interleukin-3 (IL-3) regulated (NFIL3) also known as E4 promoter-binding protein 4 (E4BP4)
Rora	Retinoic-acid-receptor-related orphan receptor-α (RORα)
Gata3	GATA binding protein-3 (GATA-3)
Tcf7	T cell factor 1 (TCF-1)
Gfi1	Growth factor independent 1 (Gfi1)
Rorct	Retinoic-acid-receptor-related orphan receptor-γt (RORγt)
Ikzf1	IKAROS family zinc finger 1 (Ikaros)
Pax5	Paired box 5
Ptcra	Pre T-cell antigen receptor alpha