Innate Lymphoid Cell Development: A T Cell Perspective

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Abstract

Innate lymphoid cells (ILCs) and NK cells have garnered considerable interest due to their unique functional properties in immune defense and tissue homeostasis. Our current understanding of how these develop has been greatly facilitated by knowledge of T cell biology. Models of T cell differentiation provided the basis for a conceptual classification of these innate effectors and inspired a scheme of their activation and regulation. In this review, we discuss NK cell and ILC development from a 'T cell standpoint', in an attempt to extend the analogy between adaptive T cells and their innate ILC and NK cell counterparts.

Introduction

The discovery some 40 years ago of innate cytotoxic cells (natural killer, NK) in mice and man (Kiessling et al., 1975; Herberman et al., 1975) forced immunologists to rethink several concepts about lymphocyte development and function. While similar to cytotoxic T lymphocytes (CTL) in their capacity to eliminate target cells, NK cells differ dramatically in the way they 'sensed' target cells (as they lack a T cell receptor) and in their kinetics of action. Molecular mechanisms that are responsible for this divergence are well described (Raulet and Vance, 2006). Nevertheless, the immune functions of these two diverse lymphocyte subsets appear analogous, reflecting the conservation of an underlying developmentally-driven process (Sun and Lanier, 2011).

Comparing 'innate' and 'adaptive' lymphocytes has intensified over the past decade with the discovery of an extended, related family of innate lymphoid cells (ILC). Similar to T cells of the adaptive immune system, ILCs are derived from common lymphoid progenitors (CLP) and display a lymphoid morphology. As is the case with NK cells, ILCs do not express a T-cell receptor (TCR) and do not require recombination machinery in order to mature. ILC function is rather controlled via activation signals in their immediate environment, resulting in copious cytokine production (Morita et al., 2016; Mjösberg and Spits, 2016). As such, ILCs are thought to play important protective roles in the early immune response against infection and cellular transformation, especially at epithelial barrier surfaces (Neill and Flynn, 2018). On the other hand, dysregulated ILC function has been implicated in several inflammatory diseases (McKenzie et al., 2014; Sonnenberg and Artis, 2015).

Three main groups of ILCs have been initially identified having remarkable functional parallels with known helper T (T_h) cell subsets (Spits and Di Santo, 2011). In each case, distinct transcription factors (TF) are expressed as key regulators of the specific cytokine production profiles; this knowledge base had been built upon years of work by T cell immunologists. A unified nomenclature rapidly emerged (Spits et al., 2013): group 1 ILC (ILC1) were defined as ILCs expressing *Tbx21* (encoding the T-box transcription factor, T-bet) and able to produce interferon gamma (IFN-γ) (Bernink et al., 2013; Vonarbourg et al., 2010), group 2 ILC (ILC2) as ILCs

expressing high amounts of *Gata3* (encoding the GATA binding protein 3, GATA3) and producing interleukin (IL)-5 and IL-13 (Mjösberg et al., 2011; Moro et al., 2010; Neill et al., 2010; Price et al., 2010), and group 3 ILC (ILC3) as those expressing *Rorc* (encoding the RAR-related orphan receptor gamma, RORγt) and producing IL-17A and IL-22 (Cella et al., 2009; Cupedo et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). As such, these recently described ILC subsets can be considered as 'innate' versions of adaptive $CD4^+$ T_h cells. Comparing these ILC subsets with conventional NK (cNK) cells show remarkable developmental similarities as well as similar rapid effector functions, placing NK cells and ILCs together as a related family of innate effector lymphocytes. Nevertheless, several properties of cNK cells set them apart from other ILC subsets (Daussy et al., 2014; Klose et al., 2014; Robinette et al., 2015), and rather suggest a distinction between 'helper' ILCs (ILC1, ILC2 and ILC3) and 'cytotoxic' ILCs (NK cells) that parallels the $CD4^+$ T_h cells versus $CD8^+$ CTL duality (Artis and Spits, 2015). The recent identification of intestinal IL-10-producing regulatory ILCs (Wang et al., 2017) may represent an additional branch of 'helper' ILCs that represent innate versions of IL-10 producing $CD4^+$ T cells (Gabryšová et al., 2018).

It is clear that the substantial knowledge of mechanisms controlling T cell differentiation and function has been particularly helpful in rapidly advancing our understanding of ILC biology. While an evolutionary relationship between T cells and ILCs still remains speculative (Vivier et al., 2016), the orchestration of T_h cell and ILC subset differentiation is remarkably shared; many TFs previously identified as key regulators in T_h cells are now known to be involved in ILC and NK cell differentiation (Fang and Zhu, 2017; Serafini et al., 2015). We will not attempt to comprehensively re-review these subjects here, but we will rather ask the question: what more can we learn about the process of ILC and NK cell development and function through analogy with the well described pathways of adaptive T cell development? Schematically, the generation of mature αβ T cells is achieved through three major stages, several of which occur in the thymus. These include a first step of T lineage commitment, where lymphoid precursors are restricted to the T cell fate. A second step – T lineage choice – selects major histocompatibility complex (MHC) class II-restricted $CD4^+$ and

MHC class I-restricted $CD8^+$ T cell lineages that produce naïve T cells that leave the thymus. A final stage of T cell clonal expansion occurs in secondary lymphoid tissues (lymph nodes) and generates functionally specialized T_h cells and CTL that participate in adaptive T cell responses and provide immune memory (**Figure 1**). In this review, we propose to apply this 'T cell-centric' view to analyze the critical stages of ILC and NK cell differentiation, highlighting the similarities but also discussing the important differences.

Commitment of Lymphoid Precursors to the ILC Fate

CLPs have been shown to have the potential to generate all known lymphocyte subsets (ILC, T, B and NK cells) following *in vivo* adoptive transfer in mice and using *in vitro* stromal cell-based growth assays in humans (Kondo et al., 1997). These multi-potent lymphoid precursors subsequently undergo stepwise restriction to individual lymphocyte cell fates. TFs are proteins able to enhance or inhibit the expression of target genes, and thereby promote certain lymphocyte lineages at the expense of others (Rothenberg et al., 2016). As such, TFs play a fundamental role in guiding the development and differentiation of the immune cells during hematopoiesis. Intrathymic signals promote this process during T cell development, generating T lineage committed precursors. For the development of ILC subsets and NK cells, core pathways that play analogous roles have been identified (**Figure 2**). We have previously used the term common ILC precursors (CILCP) to denote lymphoid precursors that have lost B and T cell potential, have no myeloid potential and give rise to the three main ILC groups and cNK cells at the clonal level (Serafini et al., 2015). CILCP include previously identified C-X-C motif chemokine receptor 6 $(CXCR6)^+$ α lymphoid precursors (αLP) and 'early innate lymphoid progenitors' (EILP) (Yang et al., 2015; Yu et al., 2014).

T cell factor 1 (TCF-1, encoded by *Tcf7*) plays a critical role in T lymphocyte development (Germar et al., 2011; Weber et al., 2011). *Tcf7*^{GFP} reporter mice provide the means to isolate EILP from adult mouse bone marrow (Yang et al., 2015). Using *in vitro* single cell assays, approximatively 60% of EILPs are able to generate all ILC lineages (ILC1, ILC2 and ILC3) and cNK cells at the

clonal level, while T and B cell fates are not observed. Curiously, EILPs are essentially CD127$^-$ (or IL-7R$^-$), unlike upstream precursors (CLP) and downstream progeny (see below) that are CD127$^+$ (Klose et al., 2014; Yang et al., 2015). More recent work showed that EILPs derive from CLPs (Harly et al., 2018) suggesting that rapid changes in CD127 expression accompany the process of ILC lineage commitment. Interestingly, a similar shutdown of CD127 expression also occurs during entry of early T cell progenitors (ETP) in the thymus (Schlenner et al., 2010) perhaps reflecting receptor occupancy-induced internalization or receptor shedding.

Studies on T cell development have previously shown the critical role for TCF-1 in T lineage specification and commitment from lymphoid precursors (Verbeek et al., 1995). TCF-1 is a direct target of Notch and over-expressed TCF-1 can rescue thymopoiesis and suppress B lineage development from Notch-deficient ETP (Weber et al., 2011). This latter point suggests that TCF-1 role in promoting T and ILC development involves, in part, repression of B cell fate. TCF-1 can activate other genes implicated in ILC development such as *Gata3* and *Bcl11b* (encoding the B-cell lymphoma/leukemia 11b protein, BCL11b) (Weber et al., 2011). Interestingly, these TFs are key drivers of thymopoiesis (Rothenberg et al., 2016), underlining the commonality of T and ILC lineage commitment mechanisms at the earliest stages when ETPs and CILCPs respectively emerge from CLP (**Figure 2**).

While the role for Notch signaling in CILCP generation is not known, *Tcf7* expression in these cells is consistent with transient Notch signaling (Cherrier et al., 2012) during ILC and NK cell lineage commitment. Strength and duration of the Notch signaling, combined with differential concentrations of IL-7, may also be crucial in the commitment towards T cell or ILC. Previous studies have already demonstrated that Notch is crucial for commitment towards a T cell but not a B cell fate (Radtke et al., 1999; Schmitt and Zúñiga-Pflücker, 2002), and that high Notch amounts are further required for generation of αβ T cells, when reduced amounts are sufficient for generation of γδ T cells (Washburn et al., 1997). It is also known that Notch signals are dispensable for generation of NK cells (Radtke et al., 2000) and fetal RORγt$^+$ cells (Possot et al., 2011). However, a recent study

suggests that short exposition or intermediary amounts of Notch combined with high amounts of IL-7 in CLPs leads to ILC2 generation, while sustained or high amounts of Notch signaling induces differentiation into T cells (Koga et al., 2018). Together, these data indicate that Notch acts at multiple stages of T cell differentiation and suggest that modulation of Notch signaling may be critical in T cell versus ILC fate decision.

Several TFs operate to segregate CILCPs from ETPs, allowing definitive separation of the ILC and NK lineages from T cells, including nuclear factor interleukin-3 regulated protein (NFIL3) and thymocyte selection-associated high mobility group box protein (TOX). *Nfil3*$^{-/-}$ as well as *Tox*$^{-/-}$ mice show severe reductions in mature ILC numbers (Seehus et al., 2015; Geiger et al., 2014; Seillet et al., 2014; Xu et al., 2015; Yu et al., 2014). Mechanistically, NFIL3 can bind the *Tox* promoter, and *Nfil*$^{-/-}$ CLP have decreased expression of *Tox* (Yu et al., 2014). In turn, TOX appears to be crucial for *Tcf7* expression downstream of CLP (Seehus et al., 2015), leading to a NFIL3 → TOX → TCF-1 activation cascade **(Figure 2)**. Low amounts of NFIL3 in CLPs and high expression of NFIL3 and TOX in CILCPs support such a feed-forward model that can serve to maintain TCF-1 expression in CILCPs, at least during the earliest stages of ILC and NK cell lineage commitment. This cascade appears redundant in T lineage commitment, as *Nfil3* deficient mice develop a normal T cell compartment (Xu et al., 2015). In this later case, sustained expression of TCF-1 is likely maintained through persistent Notch signaling.

As CILCPs further differentiate, expression of the TF Inhibitor of DNA 2 (ID2) cements their commitment towards the ILC fate (**Figure 2**). ID proteins (ID1 to ID4) belong to the helix-loop-helix (HLH) family of TFs that act as repressors of E proteins (E2A, E2-2 and HEB) transcriptional activators (Verykokakis et al., 2014). E proteins potently promote B and T lymphopoiesis and ID2 counteracts this function promoting development of NK cells and lymphoid tissue-inducer (LTi) cells (Heemskerk et al., 1997; Yokota et al., 1999). Further studies have demonstrated that ID2 is critical for generation of all ILC subsets (Moro et al., 2010; Satoh-Takayama et al., 2010; Cherrier et al., 2012), reinforcing the notion of ID2 as an essential mechanism for excluding B and T cell fates from

CLP and thereby assuring ILC an NK cell lineage commitment (Verykokakis et al., 2014). ID2 expression appears to enforce innate qualities to both adaptive and innate lymphocytes. *Id2* expression is up-regulated in activated and memory $CD4^+$ and $CD8^+$ αβ T cells, in γδ T cells and in invariant T cells (mucosal associated invariant T cells, invariant NKT cells). Loss of ID2 signaling in mature NK cells results in functional defects and modifies NK cell chromatin accessibility, shifting transcriptional profiles towards those observed for naïve $CD8^+$ T cells (Zook et al., 2018). Together these studies suggest that ID2 may represent the molecular switch that imprints 'rapid reactivity' in both innate and adaptive lymphocytes. Signals that up-regulate ID2 expression in CILCPs are not fully characterized, although it has been shown that NFIL3 can bind the ID2 promoter in ILC precursors (Xu et al., 2015).

As ID2 plays a critical role in ILC and NK cell development, ID2 expression offered the means to isolate and characterize early NK and ILC precursors (NKP, ILCP). Using *Id2*^{GFP} reporter mice, both $CD127^+$ pre-pro NKP and $CD127^+$ $\alpha_4\beta_7^-$ $ID2^+$ common 'helper' ILC progenitors (CHILP) have been identified (Carotta et al., 2011; Klose et al., 2014). As expected, $ID2^+$ NKP give rise to mature NK cells *in vivo* (Carotta et al., 2011), while $ID2^+$ CHILP can generate ILC1, ILC2, ILC3 and lymphoid-tissue inducer (LTi) cells but not cNK cells *in vivo* and *in vitro* (Klose et al., 2014). It is indeed curious that $ID2^+$ ILCP which can generate both cNK and ILCs have not identified, leading to the suggestion that ID2 might differentially regulate cNK versus ILC fates. Alternatively, current *Id2* reporter mice may lack sufficient sensitivity to identify these rare multipotent NK and ILC progenitors.

In summary, this first stage of ILC and NK lineage commitment shares some notable parallels with T lineage commitment through use of similar TF-driven mechanisms that initiate lineage specification and restrict alternative lymphocyte fates. The end product of this stage is a bipotent CILCP whose partner in the T lineage pathway is the $CD4^+CD8^+$ 'double-positive' (DP) thymocyte (**Figure 2**). We propose that these two distinct cell types (DP cells and CILCPs) have analogous functions as they provide the cellular substrate for 'helper' and 'killer' lineages in the adaptive and

innate immune systems, respectively.

Establishing 'helper' versus 'killer' ILC Lineages

$CD4^+$ and $CD8^+$ T cells provide complementary 'helper' and 'cytotoxic' functions during adaptive immunity. These two αβ T cell lineages are generated from DP thymocytes in order to assure MHC reactivity ($CD4^+$ T cells - MHC class II, $CD8^+$ T cells – MHC class I) and eliminate self-reactivity (central tolerance). The end result of this critical checkpoint is the generation of naïve T cells that can differentiate into 'helper' and 'cytotoxic' T cells upon triggering by cognate antigen in secondary lymphoid organs (**Figure 1**). Does an analogous process occur in the innate immune system to generate naïve ILCs and NK cells? NK cell differentiation is clearly influenced by interactions with MHC ('licensing') through MHC class-specific receptors that leads to 'education' by 'self' (for examples, see Raulet and Vance, 2006). It is not clear whether a similar process occurs during ILC development, but the general absence of MHC-specific receptors on ILCs makes this unlikely. Still, despite the absence of an obligatory directive role for MHC in ILC or NK cell development, there is clear evidence in the literature for segregation of 'helper' ILC and 'cytotoxic' NK lineages via restricted progenitors, suggesting that other non-MHC mechanisms (including TFs) may drive this process.

The TF GATA-3 has multiple roles in development of hematopoietic and non-hematopoietic tissues. Within immune cells, GATA-3 is essential for early T cell development and promotes $CD4^+$ T cell lineage choice in the thymus and T helper-2 (T_h2) cell differentiation in the periphery (Tindemans et al., 2014). GATA-3 ablation severely affects development of all ILC subsets (ILC1, ILC2, ILC3) (Hoyler et al., 2012; Serafini et al., 2014; Yagi et al., 2014) but is not essential for cNK development (Samson et al., 2003). These observations suggest that GATA-3 operates in the ILC lineage (but not the NK lineage) downstream of CILCP to generate CHILP. As such, GATA-3 appears to operate as a central regulator of 'helper' lineage choice in both innate and adaptive lymphocytes. Previous studies have documented that GATA-3 helps repress B lineage potential in ETPs (García-

Ojeda et al., 2013). This may occur in part through negative regulation of TFs that promote the B lineage program, such as early B-cell factor 1 (EBF-1). Interestingly, EBF-1 promotes B cell differentiation from CLPs via repression of ID2 and TCF-1 (Nechanitzky et al., 2013) documenting the mutual antagonism inherent in the B versus ILC and T pathways.

A similar observation has been made concerning the TF pro-leukemic zinc finger (PLZF, encoded at *Zbtb16*). Using *Zbtb16*^{GFP} reporter mice, a PLZF^{+} ILC precursor population has been identified in fetal liver and adult bone marrow that can give rise to ILC1, ILC2 and NKp46^{+} ILC3, but has apparently lost potential for LTi cells and cNK cells (Constantinides et al., 2014). A fraction of CHILP express PLZF (Klose et al., 2014) and GATA-3 is co-expressed in PLZF^{+} ILCP (Constantinides et al., 2014). Interestingly, *Zbtb16* is only transiently expressed during ILC development, and absent in mature ILCs suggesting a stage-specific function. Single-cell RNA sequencing of CD127^{+} bone marrow lymphoid precursors leads to the discovery of a PD-1^{+} ILCP with similar characteristics (diminished capacity to generate LTi and CD49b^{+} cNK cells) (Yu et al., 2016) providing a simple means to prospectively isolate CHILPs.

How 'cytotoxic' NKPs and 'helper' ILCPs are differentially generated is largely unknown, but one may consider analogies with the process of intrathymic CD4^{+} and CD8^{+} T cell lineage choice. In the thymus, DP cells undergoing persistent TCR signaling are thought to upregulate expression of *Zbtb7b*, the gene coding for the T-helper-inducing POZ/Krüppel-like factor (ThPOK) (Sun et al., 2005). This zinc finger TF belongs to the same family as PLZF, and works to antagonize *Cd4* silencers as well as *Cd8* enhancers, and suppresses Runt-related transcription factor 3 (RUNX3) expression that is critical for CD8 lineage choice (Muroi et al., 2008). The role for ThPOK in ILC development is not known although it is expressed by several ILC subsets. Regulation of *Zbtb7c* expression (or that of other *Zbtb* family members) in multipotent NK and ILC progenitors may allow for CHILP versus NKP lineage choice by an analogous mechanism.

In the 'kinetic signaling' model (Singer et al., 2008), CD4 versus CD8 lineage choice in the thymus is governed by persistence of TCR signals (that promote CD4^{+} cells) versus cytokine signals

(that promote $CD8^+$ cells). The principle downstream target of cytokine signaling at this stage is RUNX3 that orchestrates the 'cytotoxic' lineage gene signature (Singer et al., 2008), although RUNX1 is also involved (Etzensperger et al., 2017). Given the important role for RUNX TFs in NK cell development (Ebihara et al., 2015), it is interesting to postulate a similar cytokine-driven mechanism to drive NKP development from CILCP. Recently, a series of cytokines (IL-6, IL-7, IL-15, IFN-γ, TSLP, TGF-β) have been identified that promote CD8 lineage choice in the thymus (Etzensperger et al., 2017). Amongst these, IL-15 is a likely candidate that can favor the emergence of 'cytotoxic' NKP from CILCPs to help establish the NK lineage. Still, multiple cytokines (presumably derived from distinct cell types) can facilitate CD8 lineage choice in the thymus, demonstrating cytokine redundancy for the generation of the 'cytotoxic' lineage that may also apply to innate lymphocytes.

In summary, this second stage of ILC and NK cell development allows for the generation of ILC restricted precursors (including CHILPs and $PLZF^+$ ILCPs) and NK cell restricted precursors (NKP). This process bears some resemblance to intrathymic CD4 versus CD8 lineage choice that operates on DP thymocytes and produces naïve $CD4^+$ and $CD8^+$ T cells. By extension, the resultant ILCP and NKP that develop at this stage would represent the counterparts of naïve $CD4^+$ and $CD8^+$ T cells within the innate immune system (**Figure 2**).

Differentiation of functionally distinct ILC subsets

Following on from our proposed analogy between T cell developmental and ILC and NK cell development, the next step involves the conversion of naïve cells into functional effectors (**Figure 1**). For the adaptive immune system, this process generates CTL and T_h subsets, while for the innate system, NKP and CHILP would give rise to cNK cells and the various different mature ILC subsets (**Figure 2**).

The essential TFs that drive the differentiation of 'helper' ILC subsets and 'cytotoxic' NK cells have been fully characterized (**Figure 2**). NK cells and ILC1 produce type 1 cytokines (including

IFN-γ) in a T-bet-dependent fashion, as *Tbx21*$^{-/-}$ mice show cytokine production deficiencies in both these subsets (Klose et al., 2014; Townsend et al., 2004). T-bet concentration appears to differentially impact NK cell and ILC1 homeostasis since *Tbx21* is partially repressed during NK cell development and its over-expression leads to decreased numbers of cNK cells and increased number of ILC1, in particular in the liver (Daussy et al., 2014). Downstream targets of *Tbx21* in NK cells and ILC1 include *Runx3* similar to that observed in T_h1 cells (Djuretic et al., 2007). Indeed, RUNX3 expression is essential for generation of T-bet$^+$ NK cells, ILC1 and NKp46$^+$ ILC3 (Ebihara et al., 2015).

In addition to T-bet, cNK cells require several additional TFs that are not shared with ILC1. One such factor required for cNK cell development is Eomesodermin (EOMES), that is already expressed in NKPs (Male et al., 2014) but not in ILCPs. In *Eomes*$^{fl/fl}$*Vav*$^{Cre/+}$ mice, NKPs fail to mature, identifying EOMES as a positive critical regulator of cNK cell differentiation, whereas other ILC subsets are unaffected (Gordon et al., 2012; Klose et al., 2014). Other NK cell-specific TFs include the Zinc finger E-box-binding homeobox 2 (ZEB2) and the TF SMAD4 (Cortez et al., 2017; van Helden et al., 2015; Robinette et al., 2015). In NK cells, *Zeb2* is induced by T-bet, and is required for NK cell maturation and egress of the bone marrow through expression of the lysosphingolipid sphingosine 1-phosphate (S1PR5) (van Helden et al., 2015). Deficiency of SMAD4, on the other hand, lead to specific loss of CD49b$^+$ cNK cells in the bone marrow and in peripheral organs, although the underlying mechanisms are not fully understood (Cortez et al., 2017).

Concerning ILC2 differentiation, key TFs that drive this process include RORα, TCF-1, BCL11b and GATA-3 (Califano et al., 2015; Hoyler et al., 2012; Klein Wolterink et al., 2013; Walker et al., 2015; Wong et al., 2012; Yu et al., 2015). A mechanistic understanding of how these TFs promote ILC2 differentiation has not yet been obtained, although T cell research again may provide some clues. As previously mentioned, TCF-1 is a Notch target in ETP, and can induce *Gata3* and *Bcl11b* expression (Weber et al., 2011). High amounts of TCF-1 in ILC progenitors may predispose to ILC2 differentiation by priming *Gata3* and *Bcl11b* promoters. GATA-3 transcriptionally activates type two cytokines, due to its ability to bind to the *Il5* and *Il13* promoters (Zhang et al., 1997) **(Figure**

2). In contrast, BCL11b appears to influence ILC2 differentiation through regulation of *Il17rb* (IL-25R) expression (Yu et al., 2016). Together, these TFs coordinate expression of ILC2-specific effector functions and responsiveness to ILC2-dependent homeostatic cytokines thereby assuring productive ILC2 differentiation.

RORγt is essential for generation of all group 3 ILCs produced during fetal and adult life (Cording et al., 2014). *Rorc* can be activated via retinoic acid (RA) receptor signaling *in utero* as well as in adult mice (van de Pavert et al., 2014). RORγt can subsequently interact with SRC family co-activators and bind DNA for transcription activation (Ciofani et al., 2012), while RA signals ensure later migration to the gut by activating expression of gut-homing receptors such as C-C motif chemokine receptor 9 (CCR9) (Kim et al., 2015). The role for RORγt in ILC3 differentiation appears similar to that operating during T_h17 cell polarization. Indeed, RORγt mutations that cripple T_h17 polarization (following failure of Lys69 ubiquitination) also induce loss of LTi-dependent Peyer's patches (He et al., 2017).

ILC Differentiation: when and where?

While the TF mechanisms driving ILC and NK cell differentiation are being unraveled with increasing depth (Fang and Zhu, 2017; Serafini et al., 2015; Zook and Kee, 2016), our knowledge of when and where this process occurs remains unclear. The longstanding dogma states that NK cells are generated in the adult bone marrow (Kim et al., 2002), however, the presence of NKPs in the circulation and in tissues suggested an alternative model (that we termed 'NK-poiesis') whereby immature NKPs would complete their differentiation outside the bone marrow (Di Santo, 2008). More recently, circulating and tissue-resident human ILC precursors have been characterized that have the potential to generate all human ILC subsets as well as cNK cells (Lim et al., 2017). These cells have properties in common with multi-potent $CD34^+$ $ROR\gamma t^+$ ILC precursors previously identified in human tonsils (Scoville et al., 2016). Human ILCPs are small resting lymphocytes and lack the functional outputs of mature ILC or NK cells, although they are clearly ILC and NK cell lineage

committed, with expression profiles of innate lymphocytes (ID2, TOX, CD127). Additional epigenetic analysis demonstrates that these cells are poised for further differentiation, suggesting that human ILCPs were 'naïve' (Lim et al., 2017). The identification of a circulating human ILCP suggests that ILC differentiation might be possible outside of the bone marrow, prompting us to propose the term 'ILC-poiesis' which described the process of ILC generation in tissues in response to local inflammatory signals (Di Santo et al., 2017; Lim et al., 2017).

The proposed processes of 'NK-poiesis' and 'ILC-poiesis' are inspired by existing models of T cell differentiation in the adaptive immune system (**Figure 1**). In all cases, naïve cells that lack effector functions are activated to mature into functionally competent cells that can participate in immunity. For T cells, this process takes place in secondary lymphoid organs (lymph nodes, Peyer's patches) and involves antigen presentation by mature dendritic cells that triggers specific TCRs (signal 1) in the context of appropriate co-stimulation (signal 2) and cytokines (signal 3) (Curtsinger and Mescher, 2010). For ILC and NK cells, both the precise tissue location as well as the signals required to assure 'NK-poiesis' and 'ILC-poiesis' remain to be fully characterized. Still factors that are likely to be important in this process include cytokines that initiate the process (for example, IL-1β, IL-12, IL-23, IL-25, IL-33, thymic stromal lymphopoietin) and maintain activated or dividing cells (IL-2, IL-7, IL-15). The cellular sources for these factors may include sentinel cells (epithelial cells, dendritic cells, tissue macrophages) as well as stromal cells. As NKPs and ILCPs are known to be highly responsive to cytokines, 'NK-poiesis' and 'ILC-poiesis' may have an advantage of more rapid kinetics (hours) compared to T_h cell differentiation (days).

The 'NK-poiesis' and 'ILC-poiesis' models provide a mean for rapid and precisely localized immune responses. As the 'naïve' innate lymphocyte equivalents are free to circulate throughout the organism, any local stress or inflammation that is associated with infection or transformation would trigger the process of NK cells and ILC differentiation where it is required. The local response by sentinel cells and stroma will vary depending on the nature of the inciting pathogenic event; as such, the generation of the appropriate ILC or NK cell response would be 'tuned' to the environment. The

'on demand' nature of 'NK-poiesis' and 'ILC-poiesis' is therefore coordinated in time and space.

The requisite influence of environment on the type of NK cell and ILC subset generated in 'NK-poiesis' and 'ILC-poiesis' models offer the means to understand some peculiar aspects of NK cell and ILC biology. For example, NK cells and ILC subsets are differentially repartitioned throughout the organism (Chea et al., 2015; Simoni et al., 2017), yet the precise mechanisms driving this are still poorly understood (**Figure 3**). This can be partly explained by effects of temporal seeding during embryogenesis (Sawa et al., 2010), by organ-specific expression of chemokines receptors and integrins on polarized ILCs (Satoh-Takayama et al., 2014), or by maintenance signals (Yang et al., 2018). Moreover, mature ILCs are proposed to be tissue-resident cells (Gasteiger et al., 2015), with little or no capacity for migration, although this may change upon inflammation (Huang et al., 2018). The additional concept of generation of NK cells and ILC subsets from 'naïve' precursors in tissues in response to environmental signals may help explain tissue-specific differences in ILC and NK cell homeostasis. For example, adult bone marrow is rich in NK cell and immature ILC2, whereas ILC3 are rare (Hoyler et al., 2012; Sawa et al., 2010). In contrast, fetal and adult gut strongly promote ILC3 development with little or no NK cell generation (Bando et al., 2015; Cherrier et al., 2012; Klose et al., 2014). How different tissue environments program specific NK and ILC outputs under steady-state conditions remains unclear but are likely to be a recapitulation of what occurs in the context of infection and inflammation.

Fetal vs. adult ILC development

Tissue-specific ILC development occurs *in utero* in the fetal liver. This process partly resembles ILC development in the adult bone marrow with the generation of *Id2*- and *Zbtb16*-expressing CHILPs (Constantinides et al., 2014; Klose et al., 2014) as well as Arginase-1 (Arg-1)-expressing CILCPs (Bando et al., 2015). Fetal liver also harbors NKPs able to differentiate into NK cells *in vitro* (Tang et al., 2012). It therefore appears that the first two stages of ILC and NK cell development (generation of CILCPs, generation of CHILPs and NKPs) occur early in life during the

fetal period. Interestingly, fetal liver also contains a predominant ILC3 precursor that expresses the TF RORγt and the LTi surface marker CD4 (Cherrier et al., 2012; van de Pavert et al., 2014; Sawa et al., 2010). These RORγt$^+$ progenitors give rise to CD4$^+$ LTi cells that migrate to the gut to initiate Peyer's patch development, and also generate IL-22 producing NKp46$^+$ ILC3 (Sawa et al., 2010). NK1.1$^+$ cells that resemble adult hepatic ILC1 are also present in the fetal liver (Tang et al., 2012) suggesting that this tissue is selectively permissive for ILC1, ILC3 and NK cell development. In contrast, fetal gut and fetal mesentery harbors ILC2 and ILC3 but few ILC1 or NK cells (Bando et al., 2015; Koga et al., 2018). Interestingly, fetal mesentery also contains PLZF$^+$ ILCP, and might thus act as an intermediary site or reservoir for early ILC development (Koga et al., 2018).

The relative importance of fetal versus adult ILC production is largely unknown. Although fetal ILCs are critical for the generation of Peyer's patches in the gut (Eberl et al., 2004), their contribution to the adult ILC3 pool is less clear. While adult ILCs appear tissue-resident (Gasteiger et al., 2015; Huang et al., 2018), ILC3 derived from fetal and perinatal RORγt$^+$ precursors persist for about 8 weeks (Sawa et al., 2010) (**Figure 3**). Fetal ILC and NK cell waves may nonetheless play an important role in tissue remodeling and immune protection. Renewal of ILCs in some tissues indicate that cells are recruited throughout adult life (Huang et al., 2018), perhaps through rare seeding of bone marrow ILCP and subsequent 'ILC-poiesis'. These different mechanisms likely work together to maintain ILC homeostasis in peripheral tissues of adult animals.

Role of the environment in ILC maintenance and plasticity

ILC activation in peripheral tissues relies on a 'cross-talk' with stromal and other immune cells. IL-33, for example, is a potent ILC2 activator, and can be produced by immune cells including mast cells or macrophages, as well as necrotic cells (Chang et al., 2011; Lüthi et al., 2009; Shimokawa et al., 2017). IL-1β and IL-23, on the other hand, induce activation of ILC3 and can be produced by macrophages or dendritic and epithelial cells, respectively (Macho-Fernandez et al., 2015; Mortha et al., 2014). These activating cytokines appear important for ILC homeostasis in peripheral tissues. For

example, deficiencies in *Il33* or in its receptor reduce ILC2 numbers in the lung (Stier et al., 2018). In addition to subset-specific signals, sustained IL-7 and IL-15 produced by non-hematopoietic cells is essential for the maintenance of $CD127^+$ ILCs and NK cells respectively, even after development (Robinette et al., 2017; Schluns et al., 2004; Yang et al., 2018). Stromal cells can also release metabolites (oxysterols) sensed by G-protein coupled receptors on ILCs that can exert a chemotactic effect; this has been shown to allow ILC3 to cluster within cryptopatches (Emgård et al., 2018). Together, these data reveal the important role of environmental signals in coordinating ILC homeostasis within tissues.

The preferential positioning of ILCs at mucosal sites rapidly raises the question of the role for microbial communities in the biology of these innate immune effector cells. Germ-free mice or mice treated with antibiotics display disrupted ILC populations in the gut (Gury-BenAri et al., 2016; Sanos et al., 2009; Satoh-Takayama et al., 2008). Interestingly, the percentage ILC3 is increased in germ-free mice, and ILC1 and ILC2 start expressing ILC3-specific enhancers (Gury-BenAri et al., 2016). How this is achieved mechanistically is unclear, but it is known that gut ILC3 do not require bacterial adherence to the epithelium to be affected by microbial signals (Atarashi et al., 2015). Microbiota may indirectly affect ILCs, via modulation of other immune cells or epithelial cells. Intestinal flora drives cytokine expression within epithelial cells that can activate or repress gut ILC3 activity (Mortha et al., 2014; Sawa et al., 2011). Sensing of bacterial elements by other immune cells, including macrophages, can as well be involved in ILC3 activation (Mortha et al., 2014). Alternatively, microbiota may directly impact on ILC3s via the metabolites it produces; for example, butyrate modifies $CCR6^+$ ILC3 and $NKp46^+$ ILC3 homeostasis in a context-dependent fashion in the gut (Kim et al., 2017).

Dietary metabolites can also have a direct impact on tissue ILC homeostasis. $NKp46^+$ ILC3 subsets highly express the aryl hydrocarbon receptor (AHR), a TF that can be bound and activated by plant-derived polyphenols (Kiss et al., 2011). Deletion of this TF induces increased apoptosis in $NKp46^+$ ILC3s but not in other ILC subsets (Qiu et al., 2012), and administration of a diet devoid of

phytochemicals to mice reduces ILC3 number and proliferation in the gut (Kiss et al., 2011). In a similar manner, vitamin A deficiencies have been shown to induce type 2 immunity and to modify the balance between ILC2 and ILC3, probably due to a lack of signaling through the RA receptor that is expressed in ILC3s but not in ILC2s (Spencer et al., 2014). Together, these data reveal an important role of dietary metabolites in preserving the ILC homeostatic balance in the gut.

Stability and Plasticity of ILC Effector Functions

While extracellular cues (including cytokines) condition the initial differentiation of NK cells and ILC subsets, additional signals are required to maintain a stable effector phenotype thereby restricting functional plasticity (**Figure 4**). This may be achieved through pioneer TF creating transcriptional cascades with inherent stability as it is the case with T-bet and RUNX3, or TCF-1 and GATA-3 (Djuretic et al., 2007; Weber et al., 2011). Alternatively, cross-inhibition of TF targets may stabilize effector phenotypes as it is the case for GATA-3 in ILC2 that binds the *Rorc* locus, inhibiting its expression and preventing up-regulation of ILC3-associated genes (Wei et al., 2011; Zhong et al., 2016). In T_h2 cells, T_h1-associated gene repression appears to be dependent for most of the genes on the formation of a complex between GATA-3 and BCL11b (Fang et al., 2018), and one can reasonably think that a similar complex occurs in ILC2 to suppress ILC1 signatures.

Despite subset-specific TF expression and apparently stable chromatin modifications, ILC subsets can show remarkable plasticity under appropriate circumstances (**Figure 4**). ILC3 may be the most plastic cells of their family, similar to T_h17 cells (Stockinger and Omenetti, 2017). In mice, $CCR6^-$ $NKp46^-$ ILC3 up-regulate NKp46 expression after IL-12 induced T-bet activation (Rankin et al., 2013), eventually losing expression of the subset-specific TF RORγt to become so-called "ex-ILC3 ILC1" (Vonarbourg et al., 2010). Interestingly, group 3 to group 1 plasticity can lead to pathology, as depletion of these plastic cells could reduce severity of induced colitis, mirroring the detrimental role of "ex-T_h17 T_h1 cells" (Buonocore et al., 2010; Jostins et al., 2012; Vonarbourg et al., 2010). Of note, expression of NKp46 appears to be reversible in ILC3, and controlled by a balance

between Notch and TGF-β signaling (Verrier et al., 2016; Viant et al., 2016). In humans, plasticity between group 1 and group 3 has been observed with IL-12 inducing IFN-γ production in ILC3 and IL-1β and IL-23 inducing IL-22 in ILC1 (Bernink et al., 2015).

ILC2 can also exhibit functional plasticity (**Figure 4**). Under stimulation with IL-12, ILC2 express IFN-γ, in both mouse and human models (Bal et al., 2016; Lim et al., 2016; Ohne et al., 2016). ILC2 plasticity can moreover by primed by IL-1β signaling. Indeed, this potent ILC2 activator also induces low amounts of *Tbx21* and *Il12rβ*, making ILC2 more susceptible to extra-cellular signals driving plasticity (Ohne et al., 2016). Similarly, murine ILC2 can acquire IL-17-producing capacity after IL-25 treatment, thus contributing to clearance of fungal infections (Huang et al., 2015). In these cases, 'plastic' ILC2 retain IL-13 expression, indicating that they only partially switch their phenotype. With the recent description of IL-9 producing ILC2 (Rauber et al., 2017), these data suggest very similar plastic properties between ILC et T_h cell subsets (Murphy and Stockinger, 2010). Still, ILC1 and ILC2 are relatively stable subsets (compared to ILC3) due to GATA-3 auto-activating loop and mutual exclusion between T-bet and GATA-3 (Hwang et al., 2005; Ouyang et al., 2000). This may also explain why plastic ILC2 do not completely lose type 2 properties.

Epigenetic states control ILC functional stability and plasticity. The VHL E3 ubiquitin ligase targets H3K4 tri-methylation (a permissive chromatin status) of the *Ilrl1*, *Il5* and *Gata3* genes (Li et al., 2018), thus maintaining ILC2 in a 'ready-to-go' state. In NK cells, the long non-coding RNA Rroid operates similarly to control H3K27 acetylation and H3K36 tri-methylation at the *Id2* locus maintaining NK cell homeostasis by limiting apoptosis (Mowel et al., 2017). In contrast, deficiency in the lysine methyltransferase G9a induces a decrease in ILC2, due to permissive methylation near genes generally associated with ILC3 such as *Rorc*, *Il22* and *Il17f* (Antignano et al., 2016) and subsequent ILC3 fate conversion. Taken together, these data indicate analogous roles for epigenetics in ILCs and in T cells, where histone modifications are known to be crucial for balancing naïve T cell polarization and ensuring subset maintenance (Wilson et al., 2009), through shared ILCs and T_h cell regulome signatures (Koues et al., 2016; Shih et al., 2016; Sciumè et al., 2017).

Regulatory ILCs: a distinct subset?

Regulatory T cells (Treg) are characterized by expression of the TF forkhead box P3 (FOXP3) and mediate immune suppression through a variety of mechanisms, including production of anti-inflammatory cytokines, cytolysis and competitive consumption of IL-2 (Ohkura et al., 2013). Despite sensitive systems for assessing FOXP3 expression, $FOXP3^+$ ILC have not been reported. However, as FOXP3 is not required for expression of anti-inflammatory cytokines such as IL-10 (Gavin et al., 2007), regulatory ILC may still exist that operate in the context of immunity and tissue homeostasis. NK cells have been shown to produce IL-10 *in vivo* during viral or bacterial infection (Lee et al., 2009; Perona-Wright et al., 2009). Cytokines that induce IL-10 production by NK cells include IL-12, IL-15 and IL-21 (Brady et al., 2004; Burrack et al., 2018; Perona-Wright et al., 2009). Interestingly, systemic but not local infection with *Yersinia pestis* is also able to drive NK cell IL-10 production (Perona-Wright et al., 2009), suggesting that elevated cytokine concentrations are required *in vivo*. ILC2 are also capable to secrete IL-10. At steady-state, ILC2 express more *Il10* transcripts than other ILC subsets (Gury-BenAri et al., 2016). Mice treated with IL-33 or papain further increases *Il10* expression in lung ILC2 (Seehus et al., 2017). In culture, IL-2 and RA act synergistically to potentiate IL-10 release by ILC2, while TGF-β displays an inhibitory effect (Maroof et al., 2008).

Recently, a regulatory ILC subset has been described (Wang et al., 2017). This $CD127^+CD90^+CD25^+$ population is detected in the small and large intestine, expresses IL-10 but lacks *Rorc* or *Gata3* expression. ILC_{reg} fail to express *Foxp3* but their development requires ID3. Interestingly, ID3 is expressed in differentiated Treg cells (Maruyama et al., 2011) and is also over-expressed in IL-10-producing ILC2 (Seehus et al., 2017). The biological roles for regulatory ILCs remain unclear but these cells appear to have potent capacity to suppress intestinal inflammatory responses *in vivo* (Wang et al., 2017).

Concluding remarks

Over the past 10 years, our in-depth knowledge of T cell differentiation allowed for an

accelerated ‘learning curve’ with respect to our understanding of ILC development and ILC function in normal and pathological conditions. From the original definition of NK cells as the innate counterpart of CTL, until the unified ILC nomenclature mirroring the T_h cell classification, the ‘rules’ of T cell biology have provided a focal point to compare and contrast ILC and NK cells. Recent advances in ILC biology, including a deeper understanding of the TFs networks, the description of circulating progenitors in humans, and the evidence for a regulatory ILC subset underline the strong analogies between ILC and T cells, stemming from an apparently shared model of lineage commitment, lineage choice and differentiation. Much remains to be learned about key aspects of ILC biology, especially how the dichotomy between cytotoxic NK cells and helper ILC subsets are mechanistically defined and immunologically relevant. Key questions remain unanswered: Where in the fetal liver and the bone marrow are ILC progenitors localized? How do these precursors interact with their niche? How important are fetal versus adult ILC and NK cell waves? What signals orchestrate ILC differentiation in different tissues and their retention over the lifetime of an organism? The exponential development of single-cell based technologies may provide the tools to better comprehend ILC differentiation and progenitor diversity, bypassing the technical limitations imposed by these rare populations. The latest advances in T cell imaging should likewise stimulate additional avenues of ILC investigation. Despite their obvious differences, the immune lifestyles of T cells, ILC and NK cells appear more than ever to walk the same path.

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Figure Legend

Figure 1. Analogies between ILC and T cell differentiation

Innate lymphoid cells (ILC) and T cells differentiate from hematopoietic stem cells (HSC) via a common lymphoid progenitor (CLP) in bone marrow. The cell commitment of T cell and ILC appears in the thymus or bone marrow respectively. Downstream of CLP, the generation of early T cell progenitor (ETP) and the common ILC progenitor (CILCP) initiate the branches of T cell and ILC families, respectively. Lineage choice in the thymus operates on double positive (DP) cells to generate the single positive (SP) $CD4^+$ and $CD8^+$ T cell lineages. Similarly, NK progenitors (NKP) appear and common helper ILC (CHILP) and $PLZF^+$ ILC progenitors (ILCP) differentiate from CILCPs to provide the innate counterparts. 'Cytotoxic' $CD8^+$ T cells (CTL) and 'helper' $CD4^+$ (T_H) cells differentiate from naïve $CD8^+$ and $CD4^+$ T cells in secondary lymphoid organs (SLO), while NK cells and ILC subsets may differentiate in tissues from 'naïve' NKP, CHILP and ILCP. Mature innate and adaptive effectors cells can then provide cytotoxic and helper functions in tissues, some of which remain 'resident' and/or 'memory'.

Figure 2. ILC and T cell commitment and differentiation

Progressive differentiation of T cell and innate lymphoid cell (ILC) progenitors through the action of specific factors (TFs). A non-exhaustive list of the main TF is presented. Common lymphoid progenitors (CLP) generate early T cell progenitor (ETP) and the common ILC progenitor (CILCP) in a first stage of T versus NK and ILC commitment. Subsequently, 'bi-potent' precursors (DP for T cells, CILCP for ILC and NK) undergo lineage choice to generate 'helper' and 'cytotoxic' lineages. TCF1, T cell factor 1; GATA-3, GATA binding protein 3; RUNX3, Runt related transcription factor 3; EOMES, eomesodermin; TOX, thymocyte selection-associated high mobility group box protein; ThPOK, T-helper-inducing POZ/Krüppel-like factor; ID2, DNA binding protein 2; PLZF, promyelocytic leukemia zinc finger; NFIL3, nuclear factor IL-3 induced; EOMES, eomesodermin;

AHR, Aryl hydrocarbon receptor; ID3, DNA binding 3; RORγt, retinoic acid receptor-related orphan receptor-γt; T-BET, T-box transcription factor.

Figure 3. Differential ILC 'repertoires' in tissues

Under steady state condition, ILC and NK cell distributions vary within different tissue. The mechanisms that drive these 'repertoires' remain unknown, but environmental signals are involved. Non-haematopoietic cells, such as mesenchemal cells elaborate cytokines (IL-7 and IL-15) that promote the differentiation and maturation of ILCs in fetal and adult tissues. 'NK-poiesis' and 'ILC-poiesis' from common ILC progenitor (CILCP) that occurs locally within tissues could provide one mechanism to account for these differences. Fetal ILC production (e.g; fetal liver) likely contributes to the ILC pool in adult gut.

Figure 4. ILC and T cell differentiation and plasticity

Environmental cytokines (IL, interleukin) can modulate T cells and ILC effector functions. Main pathways that are involved in differentiation and functional plasticity of 'helper' $CD4^+$ (T_h) cells and ILC are indicated. SP, single positive T cell; CILCP, common ILC progenitor.

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Figure 1

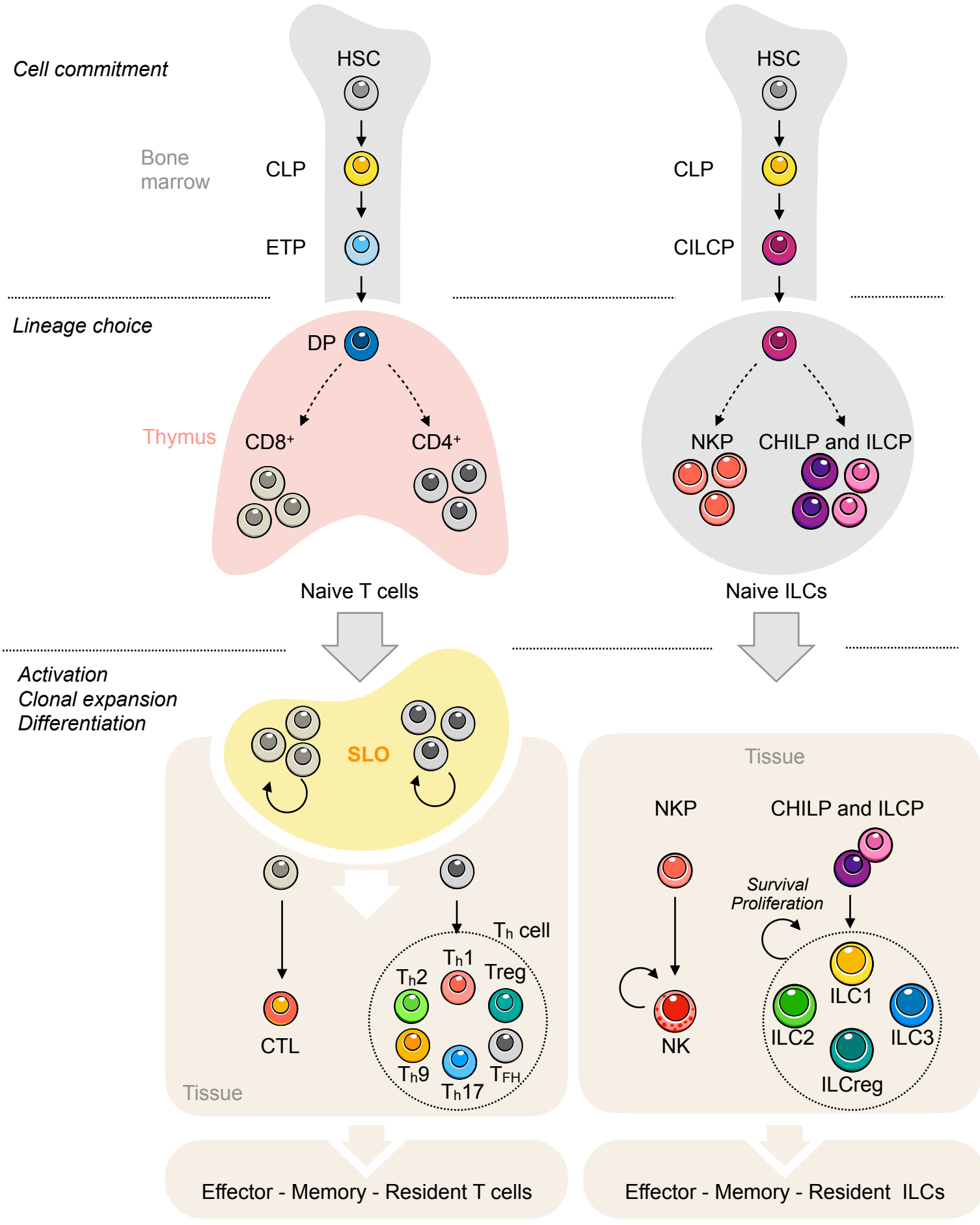

Cell commitment
HSC
Bone marrow
CLP
ETP
HSC
CLP
CILCP
Lineage choice
DP
Thymus
CD8+
CD4+
NKP
CHILP and ILCP
Naive T cells
Naive ILCs
Activation
Clonal expansion
Differentiation
SLO
Tissue
NKP
CHILP and ILCP
Survival
Proliferation
Th cell
Th1
Th2
Treg
Th9
Th17
TFH
CTL
NK
ILC1
ILC2
ILC3
ILCreg
Tissue
Effector - Memory - Resident T cells
Effector - Memory - Resident ILCs

Figure 2

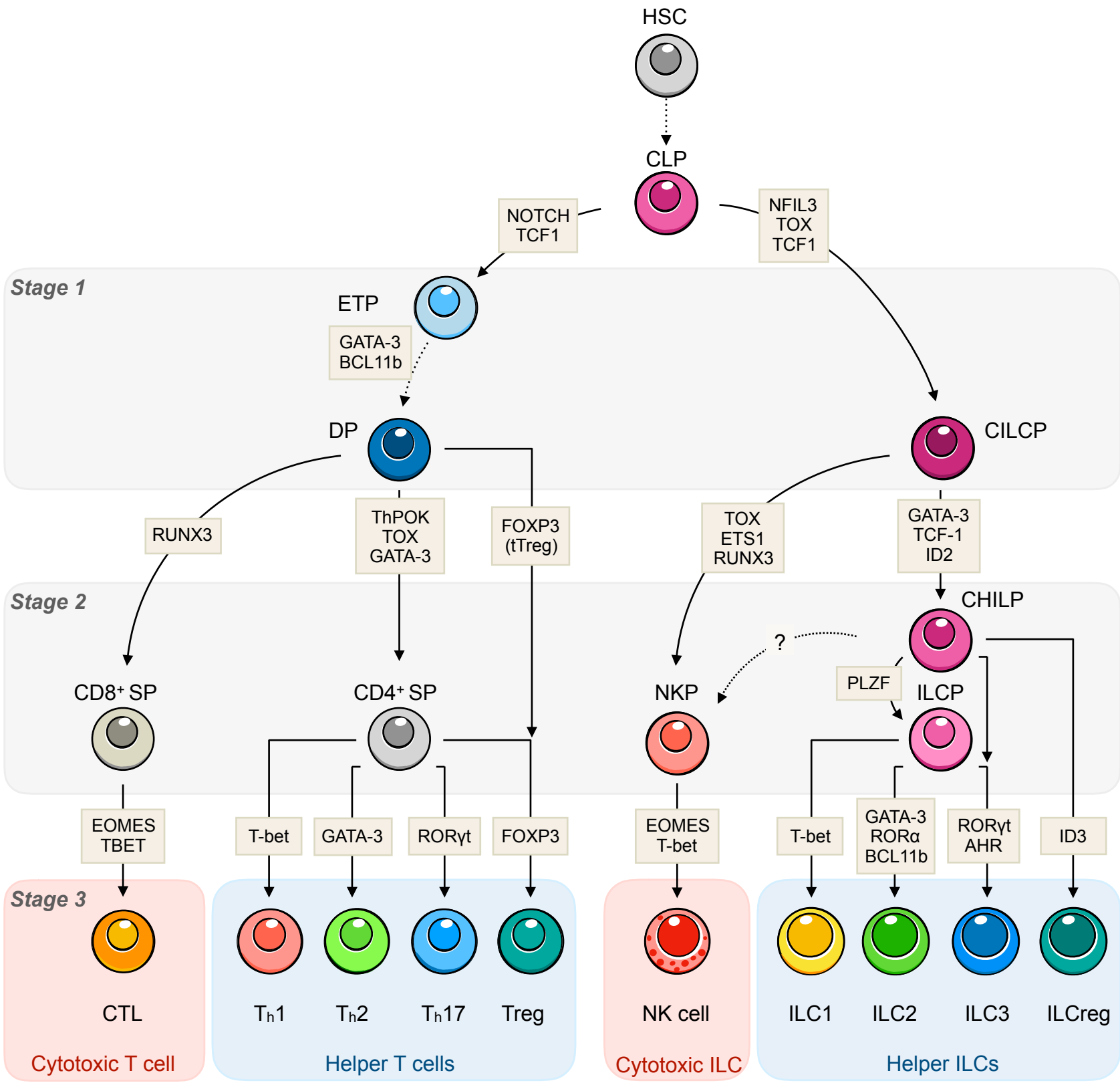

HSC
CLP
NOTCH
TCF1
NFIL3
TOX
TCF1
Stage 1
ETP
GATA-3
BCL11b
DP
CILCP
RUNX3
ThPOK
TOX
GATA-3
FOXP3
(tTreg)
TOX
ETS1
RUNX3
GATA-3
TCF-1
ID2
Stage 2
CHILP
?
PLZF
ILCP
CD8⁺ SP
CD4⁺ SP
NKP
EOMES
TBET
T-bet
GATA-3
RORγt
FOXP3
EOMES
T-bet
T-bet
GATA-3
RORα
BCL11b
RORγt
AHR
ID3
Stage 3
CTL
Th1
Th2
Th17
Treg
NK cell
ILC1
ILC2
ILC3
ILCreg
Cytotoxic T cell
Helper T cells
Cytotoxic ILC
Helper ILCs

Figure 3

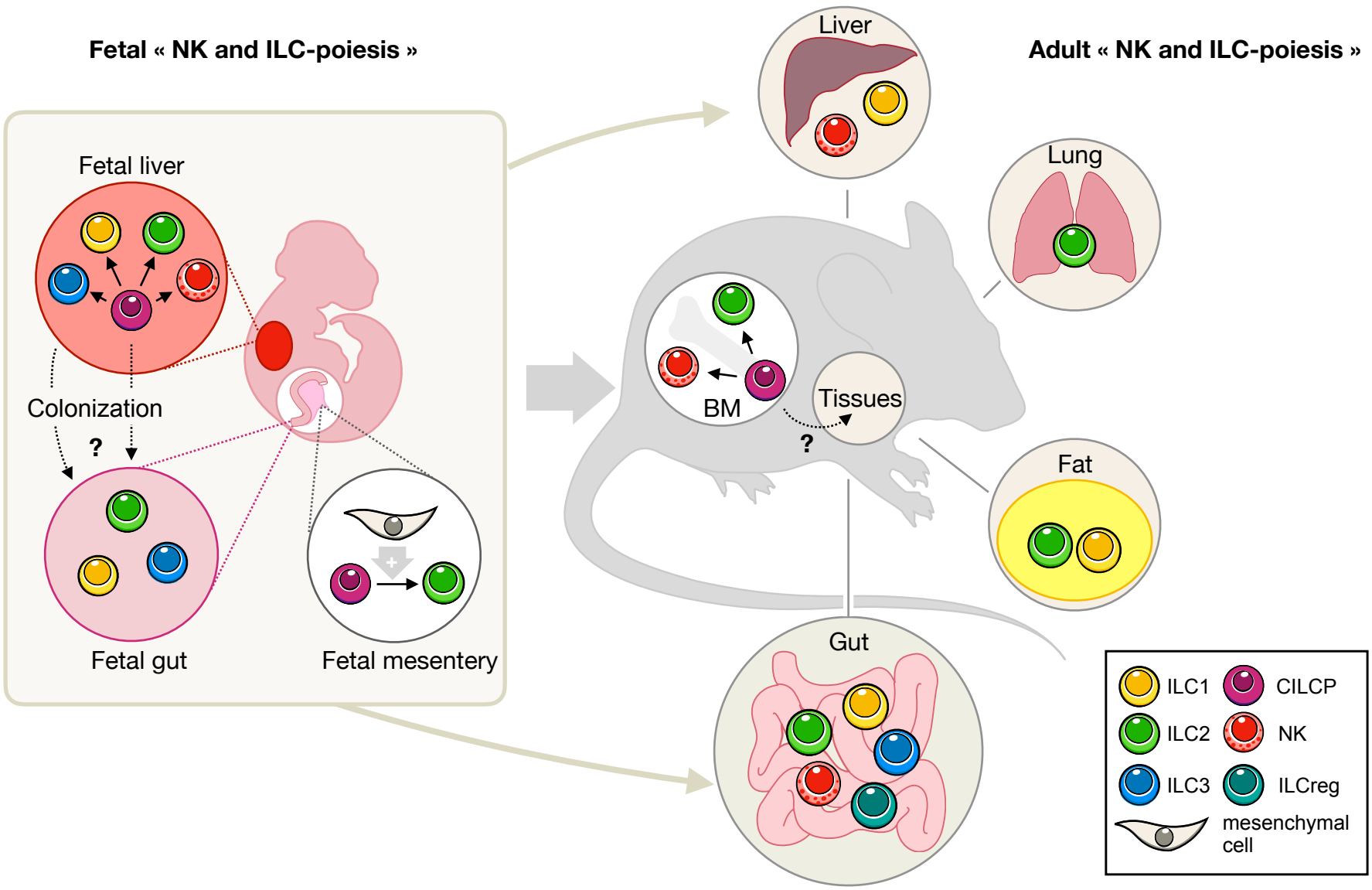
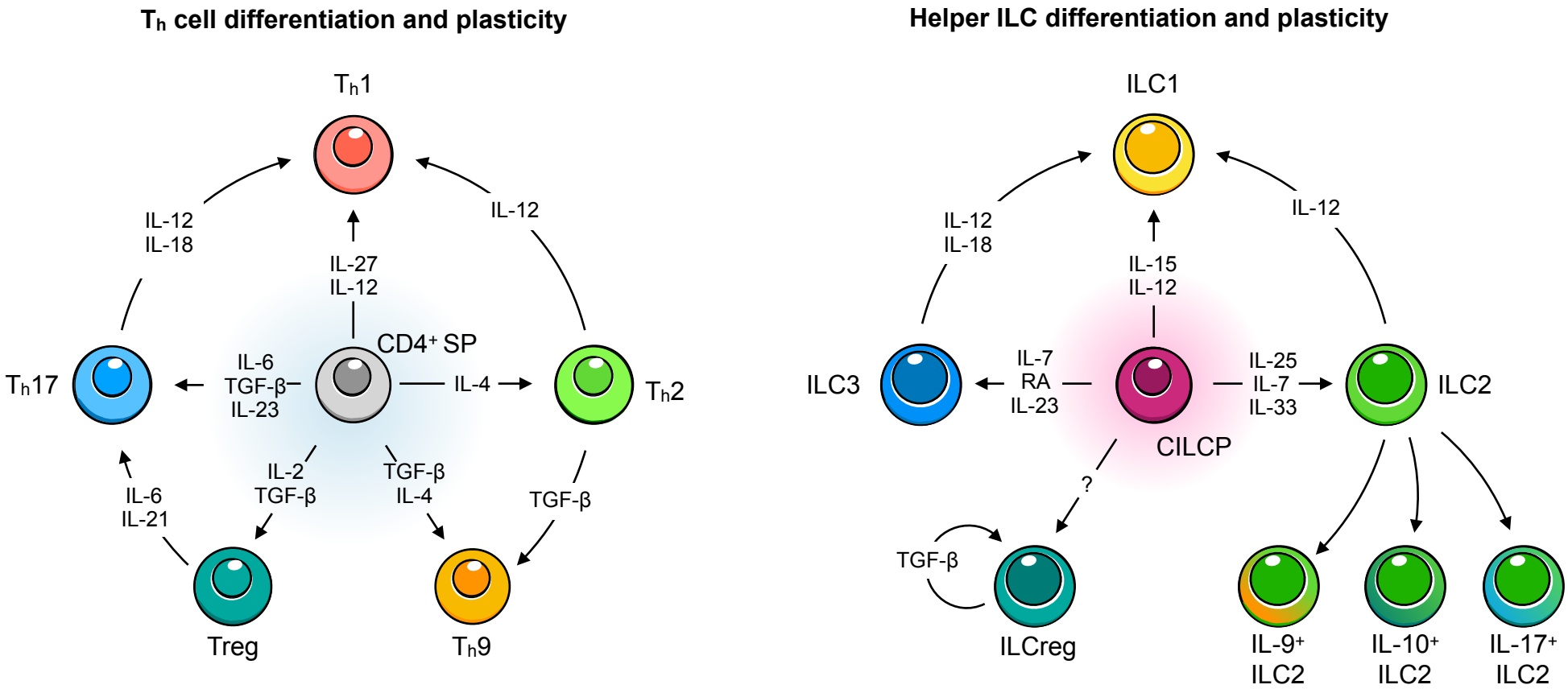

Fetal « NK and ILC-poiesis »
Adult « NK and ILC-poiesis »
Fetal liver
Colonization
?
Fetal gut
Fetal mesentery
Liver
Lung
BM
Tissues
?
Fat
Gut
ILC1
CILCP
ILC2
NK
ILC3
ILCreg
mesenchymal cell

Figure 4


Th cell differentiation and plasticity
Th1
IL-12
IL-18
IL-12
IL-27
IL-12
CD4+ SP
Th17
IL-6
TGF-β
IL-23
IL-4
Th2
IL-6
IL-21
IL-2
TGF-β
TGF-β
IL-4
TGF-β
Treg
Th9
Helper ILC differentiation and plasticity
ILC1
IL-12
IL-18
IL-12
IL-15
IL-12
ILC3
IL-7
RA
IL-23
CILCP
IL-25
IL-7
IL-33
ILC2
?
TGF-β
ILCreg
IL-9+
ILC2
IL-10+
ILC2
IL-17+
ILC2