

Development, Homeostasis, and Heterogeneity of NK Cells and ILC1

Cyril Seillet, Gabrielle T. Belz and Nicholas D. Huntington

Abstract Natural killer (NK) cells are a population of cytotoxic innate lymphocytes that evolved prior to their adaptive counterparts and constitute one of the first lines of defense against infected or mutated cells. NK cells are rapidly activated, expressing an array of germ-line encoded receptors that allow them to scan for protein irregularities on cells and kill those deemed “altered-self.” NK cells rapidly produce a broad range of cytokines and chemokines following activation by virus, bacterial, or parasitic infection and are thus key in orchestrating inflammation. NK cells have previously been viewed to represent a relatively homogeneous group of IFN- γ -producing cells that express the surface markers NK1.1 and natural killer cell p46-related protein (NKP46 or NCR1 encoded by *Ncr1*) and depend on the transcription factor T-bet for their development. Recently, a second subset of T-bet-dependent innate cells, the group 1 innate lymphoid cells (ILC1), has been discovered which share many attributes of conventional NK (cNK) cells. Despite the similarities between ILC1 and cNK cells, they differ in several important aspects including their localization, transcriptional regulation, and phenotype suggesting each subset has distinct origins and functions in immune responses. Previously, the ability to detect and spontaneously kill cells that exhibit “altered-self” which is central to tumor and viral immunity has been thought to be an attribute restricted solely to cNK cells. The identification of ILC1 challenges this notion and suggests that key contributions from ILC1 may have gone unrecognized. Thus, understanding

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the different rules that govern the behavior of ILC1 and cNK cells in immune responses may potentially open unexpected doorways to uncover novel strategies to manipulate these cells in treating disease. Here, we review recent advances in our understanding of peripheral cNK cell and ILC1 heterogeneity in terms of their development, phenotype, homeostasis, and effector functions.

Abbreviations

| | |
|-------------|--|
| α LP | $\alpha 4\beta 7^+$ lymphoid progenitor(s) |
| CLP | Common lymphoid progenitor(s) |
| CHILP | Common helper innate lymphoid progenitor |
| cNK cell | Conventional NK cell |
| IFN | Interferon |
| ILC | Innate lymphoid cell |
| IL | Interleukin |
| ILCp | ILC progenitor |
| iNK cell | Immature NK cell |
| LTi | Lymphoid tissue inducer |
| mNK cell | Mature NK cell |
| Ncr | Natural cytotoxicity triggering receptor |
| NK cell | Natural killer cell |
| RAG | Recombinase activating gene |
| Ror | Retinoic acid receptor-related orphan receptor gamma |

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1 Diversification of Innate Lymphoid Cells

NK cells were first described over 40 years ago and were identified by their ability to spontaneously lyse tumor cell lines in vitro (Herberman et al. 1975a, b; Kiessling et al. 1975a, b; Oldham and Herberman 1973). They can be found in the spleen, lymph nodes, and circulate throughout the body patrolling for infected or malignant cells but can also be found in tissues. They are characterized by their expression of the surface molecule NKp46 (encoded by *Ncr1*), NK cell inhibitory and activating receptors and their capacity to produce interferon (IFN)- γ .

More recently, additional recombinant activating gene (RAG)-independent ILC populations have been discovered. These have been classified into three main groups based on their cytokine and transcription factor expression. The ILC1 family is composed of the T-bet expressing cells and includes NK cells and ILC1 cells; ILC2 are Gata-3-expressing cells (also known as innate-helper cells, or nuocytes) originally discovered in lung, skin, or fat tissue (Price et al. 2010; Neill et al. 2010); and ILC3 that produce IL-17 and/or IL-22 and express the transcription factor Ror γ t. All three populations are made up of several subsets. This unexpected diversity reveals a whole new world of potential interactions both between ILC and adaptive immune cells in generating immune homeostasis and protection.

The ILC1 family was initially thought to include only the prototypic member, the NK cell. Diversity among NK cells, particularly in the liver, had already been noted providing the first clue that a more diverse repertoire in ILC1 existed. Indeed, during embryogenesis, lymphocytes resembling NK cells are found in the liver as early as E14 and these cells persist in the adult liver. This population appeared to be different from conventional NK (cNK) cells as they expressed distinct surface markers such as tumor-necrosis-factor-related apoptosis inducing ligand (Trail) (Takeda et al. 2005). At birth, these cells are the predominant subset in the liver but over time conventional bone marrow-derived NK cells migrate to the liver to become dominant. Until recently, these liver-derived NK cells were referred to as liver-resident NK cells or Trail⁺ NK cells due to their expression of this molecule. It is now clearer that this population represents a distinct subset of ILC that differs from cNK cells. Thus, Trail⁺ NK cells are now referred to as ILC1 although they share their dependency on the transcription factor T-bet (encoded by *Tbx21*) and their ability to produce IFN- γ (Gordon et al. 2012). These recent developments in the field highlight the diversity among NKp46-expressing cells and point toward the possibility that the different subsets contribute specific essential elements of the innate armory to protect the body.

1.1 Heterogeneity of Group 1 ILC

Group 1 ILC are defined as innate cells that lack RAG-dependent rearranged antigen receptors, express the transcription factor T-bet and produce “type I”

cytokines such as interferon (IFN)- γ (Spits et al. 2013). ILC1 have been identified in a variety of different tissue locations resulting in the identification of at least three distinct populations. These are (1) liver ILC1, (2) thymic ILC1, and (3) intraepithelial ILC1.

1.1.1 Liver ILC1

In the adult liver, cNK cells (CD49b⁺Trail⁻) coexist with CD49a⁺Trail⁺ ILC1 (Seillet et al. 2014a; Smyth et al. 2001; Takeda et al. 2005). These Trail⁺ ILC1 appear to be distinct from cNK cells as they do not appear to circulate throughout the body and maintain their residence solely in the liver at steady state (Peng et al. 2013). In addition, they differ from cNK cells as they do not require the transcription factor nuclear factor interleukin 3 (Nfil3) for development (Crotta et al. 2014; Seillet et al. 2014a). Determining whether the Trail⁺ and Trail⁻ cells represent distinct lineages have been a vexing question in the field. To shed light on this, two groups undertook transcriptomic analyses of the population and this revealed that each population exhibited a distinct gene profile (Daussy et al. 2014; Seillet et al. 2014a) (Fig. 1).

Liver ILC1 expressed a suite of molecules that were distinct from cNK cells although a core set, namely NKp46, CD122, and NK1.1, were shared. Among those molecules that were novel to ILC1 included chemokine receptor and adhesion molecules (e.g., CXCR6 and CXCR3), target lysis molecules (perforin, granzyme A/B/C and TRAIL), cytokines (TNF- α and IL-2), cytokine receptors (IL-7R, IL-17RD, IL-21R, and TGFBR), and regulatory molecules (high levels of CD200R, PD1-L, ICOSL, Lag3, and IL-2). In contrast, cNK cells express CX3CR1, CD62L, S1PR1, and S1PR5 which are not found in the ILC1. This profile could explain their lack of recirculation and establishment in the liver. These distinct profiles suggest that NK cells and ILC1 play different roles in immunity. It also highlights that in contrast to our current understanding, ILC1 probably kill target cells, albeit by mechanisms that differ from NK cells, and could be involved in regulatory roles either directly or indirectly via interactions with T cells (Campisi et al. 2011; Fallarino et al. 2004; Huang et al. 2004; Terme et al. 2012).

1.1.2 Thymic ILC1

Thymic NK cells, first described in 2006 by the group of Di Santo, differ from cNK cells as they express the IL-7 receptor α (IL-7R α , CD127) and require the transcription factor GATA-3 for development (Vosshenrich et al. 2006). In contrast, bone marrow-derived cNK cells do not required GATA-3, although it is involved in their maturation and ability to produce IFN- γ . Like liver ILC1, thymic ILC1 express low levels of Ly49 and are enriched in CD11b^{high} and CD69^{high} cells. Thymic ILC1 can develop in vitro and in vivo from the CD4⁻CD8⁻ (DN1) subset of immature thymocytes (Vargas et al. 2011) indicating that they do not develop in the bone marrow from committed precursor (Constantinides et al. 2014; Klose et al. 2014).

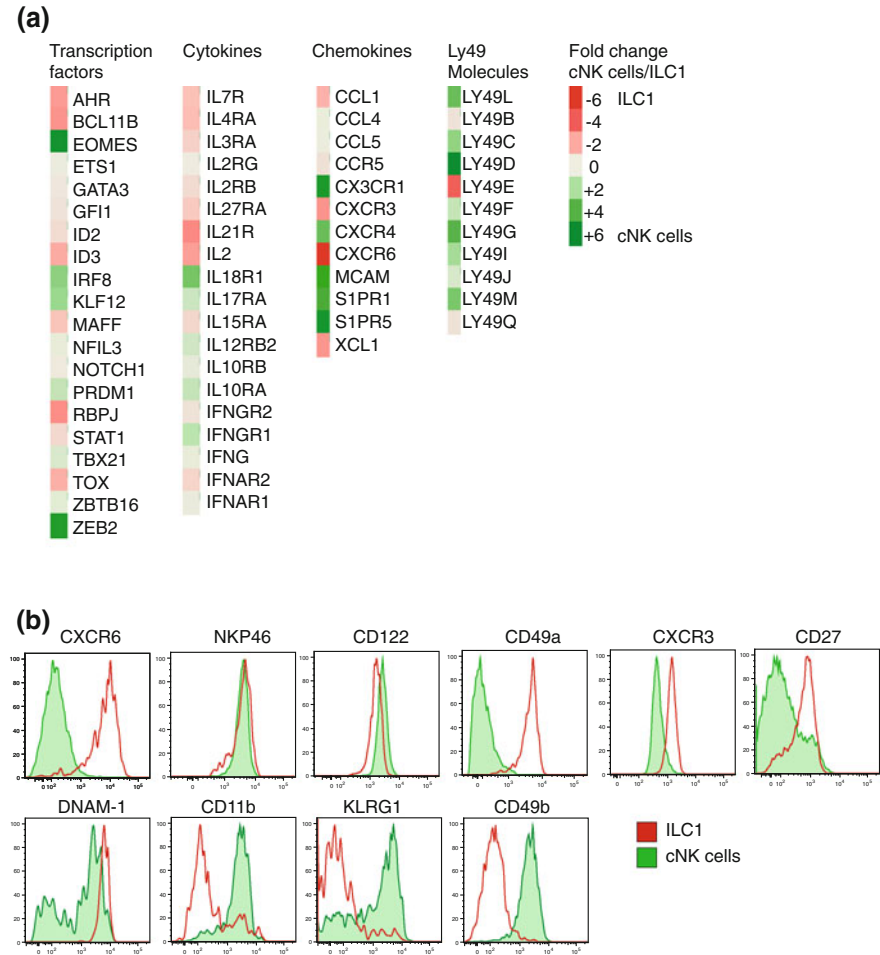


Fig. 1 ILC1 and cNK cells have distinct transcriptional signatures. **a** Heatmap showing Log2-fold change expression of selected transcription factors, chemokine receptors and ligands, cytokine receptors and ligands and Ly49 molecules as indicated, between liver cNK cells and ILC1 obtained from RNA-sequencing (*red* indicates increased expression in ILC1, *green* increased in cNK cells). **b** Flow cytometry analysis showing protein expression of indicated surface molecules on liver ILC1 (*red line*) or cNK cells (*shaded green*)

1.1.3 Intraepithelial ILC1

Two populations of intraepithelial ILC1 have been described in the intestine (Fuchs et al. 2013). The first population, unlike cNK cells, does not depend on IL-15R α for their development or survival while the second depends on T-bet but partially relies on IL-15 (Fuchs et al. 2013). Intraepithelial ILC1 have been also identified in human tonsils CD56⁺ non-T cells compartment. These are potent IFN- γ producer

and express marker such CD160, CD49a, CXCR6, CD69, and CD39 (Fuchs et al. 2013).

An additional population of poorly characterized ILC is found in the salivary gland which express both T-bet and Eomes, but are poor producers of IFN- γ and do not depend on Nfil3 to develop (Cortez et al. 2014). As yet, how these ILC1 fit into the innate landscape is unclear but overall, this diversity suggests that these populations may have specialized functional roles during an immune response and specific for the tissue in which they reside.

1.2 Development of ILC1 and Conventional NK Cells

ILC differentiation is a multistep process associated with progressive acquisition and/or down-regulation of a series of cell surface markers. They develop from common lymphoid progenitors (CLP) in the fetal liver that also gave rise to B- and T cells. In adulthood, the majority of ILC are thought to develop in the bone marrow from CLP (Huntington et al. 2007c; Possot et al. 2011). The IL-7R $\alpha^+\alpha\beta7^+$ fetal liver population was previously shown to contain progenitors with lymphoid tissue inducer (LTi) cell, T cell, NK cell, and dendritic cell, but not B cell, potential (Yoshida et al. 2001). Possot et al. (2011) identified a subset of CLP (Sca1^{low} Kit^{low} Flt3⁻ IL-7R α^+) in bone marrow and fetal liver that could differentiate into R γ ILCs, successively losing B cell and T cell potential as they acquired expression of $\alpha\beta7$ and CXCR6, respectively (Possot et al. 2011). Recently, Hooper et al. (2014) showed that this precursor could indeed clonally differentiate into all ILC lineages (Yu et al. 2014).

The common ILC progenitor: The relationship between ILC1 and cNK cells in ontogeny is not yet clear. Recently, using an approach to fate map progenitor cell development of the transcription factor promyelocytic leukemia zinc finger protein (PLZF, encoded by *Zbtb16*) identified a committed ILC precursor (ILCp) within the IL-7R $\alpha^+\alpha\beta7^+$ population in bone marrow and fetal liver. This precursor expressed high but transient levels of PLZF (Constantinides et al. 2014). Previously, PLZF has been almost solely known for its key role in NKT cell development (Kovalovsky et al. 2008; Savage et al. 2008). ILCp could generate liver ILC1, but failed to produce Eomes-expressing cNK cells. PLZF fate-mapping mice revealed that most ILC had expressed PLZF during their development but cNK cells and LTi cells had not indicating that they develop from a PLZF⁻ precursor (Constantinides et al. 2014). Although in this study, some PLZF⁺ ILCp were able to generate all three different ILC subsets, most of the precursor could generate only one or two ILC subsets suggesting that cells within this population are heterogeneous. In a second study, the common helper innate lymphoid progenitor (CHILP) population was defined as Lin⁻IL-7R α^+ CD25⁻ and was heterogeneous for PLZF expression but uniformly expressed the transcription factor inhibitor of DNA binding 2 (Id2). CHILP were able

to generate ILC2s and ILC3s, including CCR6⁺ ILC3/LTi cells, as well as small intestinal lamina propria ILC1, but not Eomes⁺ cNK cells (Klose et al. 2014).

The NK cell progenitor: The developmental intermediates downstream of the CLP leading to a committed NK cell are poorly defined. The NK precursor (NKP) has been characterized by the expression of the IL-2 receptor β chain (IL-2R β /CD122) and lack of pan-NK-cell surface markers (NK1.1 and CD49b). Only a small fraction (<10 %) of NKP, however, give rise to mature NK cells in vitro, thus it is highly likely that the population was indeed heterogeneous and only a small subset of these cells represented true progenitors (Rosmaraki et al. 2001). Using an Id2^{gfp} reporter mouse strain, it has been possible to identify a much earlier committed NK cell precursor in adult BM (Carotta et al. 2011). These progenitors were Lin⁻Id2^{high}Sca1⁺c-Kit^{int/-}Flt3⁻IL7R α ⁺ cells and by analogy to B cell development are termed pre-pro NK cells. Pre-pro NK cells efficiently generated CD3 ϵ ⁻NK1.1⁺ cells (frequency of ~1 in 2 in vitro). More recent analyses have identified a precursor committed to ILC2 lineage differentiation, the ILC2p, defined as Lin-Id2⁺Sca1^{high}CD127⁺ CD25⁺ cells. The strong phenotypic similarity between the ILCp, ILC2p, and the pre-pro NK cell suggests that pre-pro NK cells contain the common precursor for both ILCs and cNK cells.

2 Transcriptional Regulation of Conventional NK Cells and ILC1

NK cell differentiation depends on sequential steps controlled by the transcription factors Id2 (Yokota et al. 1999), nuclear factor, interleukin 3 regulated (Nfil3, encoded by *E4BP4*) (Kamizono et al. 2009; Seillet et al. 2014a), thymocyte selection-associated high mobility group box protein (TOX) (Aliahmad et al. 2010), ETS1 (Barton et al. 1998; Ramirez et al. 2012), and Eomesodermin (Eomes)(Gordon et al. 2012). How these factors cooperate is currently unclear. Several additional transcription factors have been reported to regulate NK cell maturation including GATA-binding factor 3 (GATA-3) (Vosshenrich et al. 2006), Mef (Lacorazza et al. 2002), Forkhead box protein O1 (Foxo1) (Deng et al. 2015), Ikaros family zinc finger protein 3 Aiolos (encoded by *Ikzf3*) (Holmes et al. 2014), T-box containing protein in T cells (T-bet, or Tbx21) (Townsend et al. 2004), interferon regulatory factor (Irf)-2 (Lohoff et al. 2000), and B-lymphocyte-induced maturation protein 1 (Blimp-1, encoded by *Prdm1*) (Kallies et al. 2011). Some of these transcription factors are also required for the differentiation of ILC1. For example, similar to NK cells, Gata-3 (Yagi et al. 2014) and T-bet (Daussy et al. 2014; Fuchs et al. 2013) are important for ILC1 development, although their precise roles in this process remain unclear. In contrast, other transcription factors appear to play distinctly different roles in each subset. This is highlighted by the critical dependence of cNK cells on Eomes for maturation and survival while ILC1 have no apparent reliance on this transcriptional regulator (Gordon et al. 2012; Seillet et al. 2014a).

2.1 *Inhibitor of DNA Binding 2 and E-box Proteins*

Inhibitor of DNA binding 2 (Id2) is a transcriptional regulator required at various stages of both innate and adaptive lymphocyte development and function. It is a member of the Id family (Id1-4) that shares the highly conserved helix-loop-helix (HLH) motif (Sun et al. 1991; Voronova and Lee 1994). These factors regulate transcription by inhibiting the function of E-box (or E) proteins by forming Id/E protein heterodimers to block transcription by E-proteins (Sun et al. 1991; Voronova and Lee 1994). E-box proteins are another class of transcription factor and include the family members Tcf12 (HEB), Tcf4 (E2-2), and Tcf3 (E2A) gene products E12 and E47 (Murre et al. 1989a, b). They are defined by their two highly conserved domains: (i) a HLH domain which regulates homo- or hetero-dimerization and (ii) a basic domain that is important for binding to E-box sequences on the DNA of target genes (CANNTG) (Murre et al. 1989a).

Expression of Id proteins by ILC progenitors may represent one mechanism of preventing ILC progenitors from adopting T- or B cell fate. Mature ILCs as well as $\alpha 4\beta 7^+$ ILC progenitors highly express *Id2*, and *Id2*-deficiency results in the loss of all types of mature ILCs (Yokota et al. 1999) suggesting that Id2 expression is required for ILC development. It is likely that Id2 promotes ILC development by neutralizing E-protein activity as deletion of E2A was able to partially overcome the need for Id2 in LTi cell development and could restore the development of lymphoid tissue to some degree (Boos et al. 2007). However, NK cell development in *Id2*^{-/-} *E2A*^{-/-} bone marrow was still impaired and splenic NK cell development resembled that found in *Id2*^{-/-} mice suggesting that other E-proteins might also be targets of Id2 suppression required to facilitate innate immune cell lymphopoiesis. Therefore, Id2 induction appears to be one of the first molecular steps in the induction of the ILC lineage; however, the mechanism of Id2 up-regulation in ILC progenitors remains unclear.

2.2 *Nuclear Factor Interleukin-3 (Nfil3)*

Nfil3 (nuclear factor interleukin-3; also known as E4-binding protein 4, or E4BP4) was first described as a critical transcriptional regulator for NK cell development affecting mature NK cells (Gascoyne et al. 2009; Kamizono et al. 2009) and thymic NK cells (Seillet et al. 2014a). Initial studies suggested that the blockage in NK development occurred at the transition from the NKP to the immature NK (iNK) stage but this work relied on investigations of the NKP which we now understand to be a heterogeneous population (Carotta et al. 2011). *Nfil3*-deficient mice also display a broad loss of ILC populations including ILC1, 2, and 3 together with LTi cells. This loss appears to stem from inhibition of the development of the bone marrow $\alpha 4\beta 7^+$ lymphoid progenitors and ILCPs (Seillet et al. 2014b) and thus the development of a common innate lymphoid progenitor, prior to PLZF

up-regulation. Mapping the NKP using Id2^{gfp} mice showed that deletion of *Nfil3* resulted in a significant loss of Id2⁺ cells within this progenitor stage indicating that *Nfil3* functions at the earliest steps of NK cell commitment (Seillet et al. 2014a). *Nfil3* has been proposed to induce Id2 expression because forced expression of Id2 could rescue developmental defects of NK cells from *Nfil3*^{-/-} precursors (Gascoyne et al. 2009). It is not clear that this is the case, however, as *Nfil3*^{-/-} NK cells express normal levels of Id2^{gfp} (Crotta et al. 2014; Seillet et al. 2014a). Nevertheless, *Nfil3* is not universally required for ILC differentiation as liver-resident ILC1 and those found in the skin, uterus, and salivary gland were also unaffected (Cortez et al. 2014; Seillet et al. 2014a, b; Sojka et al. 2014). Indeed, *Nfil3* appears to be only to be important early in ILC development as deletion of *Nfil3* in mature cells using NKp46^{iCre} or Rorγt^{Cre} cells has no effect on lineage maintenance or homeostasis of NK cells, or ILC3, respectively (Firth et al. 2013; Xu et al. 2015).

Although *Nfil3* appears to be critical for the commitment of CLP to the innate lineage, how it mediates this is currently not clear (Seillet et al. 2014b; Yu et al. 2014). Two alternate mechanisms have been proposed. Firstly, in the αLP population, *Nfil3* is suggested to be primed to induce ILC commitment through the induction of the transcription factor TOX (Yu et al. 2014). In the second mechanism, *Nfil3* is proposed to act on the CHILP to induce Id2 expression (Xu et al. 2015). Currently, the relationship between αLP and CHILP is not clear and whether *Nfil3* acts at two different stages in ILC progenitors is not yet clear.

Nfil3 is not required for all ILC and discrimination between different peripheral subsets has been linked to the induction of Eomes. Indeed, all Eomes-expressing NK cells, including conventional medullary and thymic NK cells, are absent in absence of *Nfil3*, whereas TRAIL⁺ NK cells that do not express Eomes appear unaffected by its loss (Seillet et al. 2014a). Ectopic expression of Eomes in *Nfil3*-deficient progenitor cells was sufficient to overcome the requirement for *Nfil3* and to restore NK cell development (Seillet et al. 2014a). In a separate study, it was shown that *Nfil3* directly bound Eomes to promote its expression (Male et al. 2014). However, despite this apparently clear delineation between *Nfil3*/Eomes dependent and independent lineages, salivary gland NK cells do not fit this pattern as they Eomes but are not lost in the absence of *Nfil3* suggesting that alternative pathways may support Eomes expression in the different ILC subsets (Cortez et al. 2014).

2.3 GATA-Binding Protein 3 (GATA-3)

A role for GATA-binding protein 3 (GATA3) was first appreciated in thymic but not cNK cells (Vosshenrich et al. 2006). Since then, it has been shown that deletion of GATA-3 in all hematopoietic cells impaired the development of all IL-7Rα⁺ ILC subsets but did not interfere with the development of cNK cells (Yagi et al. 2014). However, GATA-3 deletion induced after expression of Id2 (using Id2^{CreERT2}) led to a selective loss of both immature ILC2p in the bone marrow and consequently mature ILC2, whereas development of the Rorγt⁺ ILC3 cells was not affected

(Hoyler et al. 2012). This suggests that GATA-3 is universally required for development of all ILC subsets very early in lineage commitment prior to Id2 expression. This is supported further as the loss of GATA-3 at later stages of differentiation was dispensable for the maintenance of ILC3 subsets. Future studies will be needed to determine when GATA-3 is exactly required during ILC commitment and which GATA-3-controlled genes regulate the ILC lineage.

2.4 *T-box Factors: T-bet and Eomesodermin*

T-bet (encoded by *Tbx21*) is expressed by both ILC1 and cNK cells but these two subsets differentially depend on it for their development and maturation. The second T-box transcription factor *Eomes* (also known as Eomesodermin) is highly homologous to T-bet but its expression appears to discriminate between NK cells and ILC1 subsets. In NK cells, T-bet is required together with another T-box transcription factor Eomes to promote both cell maturation and function (Gordon et al. 2012). These two transcription factors act in a sequential manner, but T-bet directs the development of iNK cells and stabilizes the immature phenotype while Eomes induces the expression of a diverse repertoire of Ly49 receptors in NK cells and acts to maintain the mature phenotype (Gordon et al. 2012). T-bet has also been shown that T-bet directly regulates S1PR5 (sphingosine-1-phosphate receptor 5) expression required for cNK cell egress from the bone marrow (Jenne et al. 2009; Walzer et al. 2007b) and expression of IL-2R β chain expression, a component of the IL-15 receptor complex required to maintain both ILC1 and NK cells (Klose et al. 2014; Lazarevic and Glimcher 2011). ILC1 also critically depend on T-bet for their development as T-bet^{-/-} mice lack virtually all liver or intestinal ILC1s while Eomes⁺ NK cells can be found in these mice (Gordon et al. 2012; Klose et al. 2014). Analyses of Eomes reporter mice showed that at steady-state T-bet⁺Eomes⁻ cells appear to a stable population that do not subsequently give rise to T-bet⁺Eomes⁺ cells, suggesting that these cells are not immature, but represent a distinct population (Daussy et al. 2014). However, whether plasticity exists between Eomes⁺ and Eomes⁻ cells under inflammatory conditions remain to be fully elucidated.

3 Heterogeneity of Peripheral NK Cells

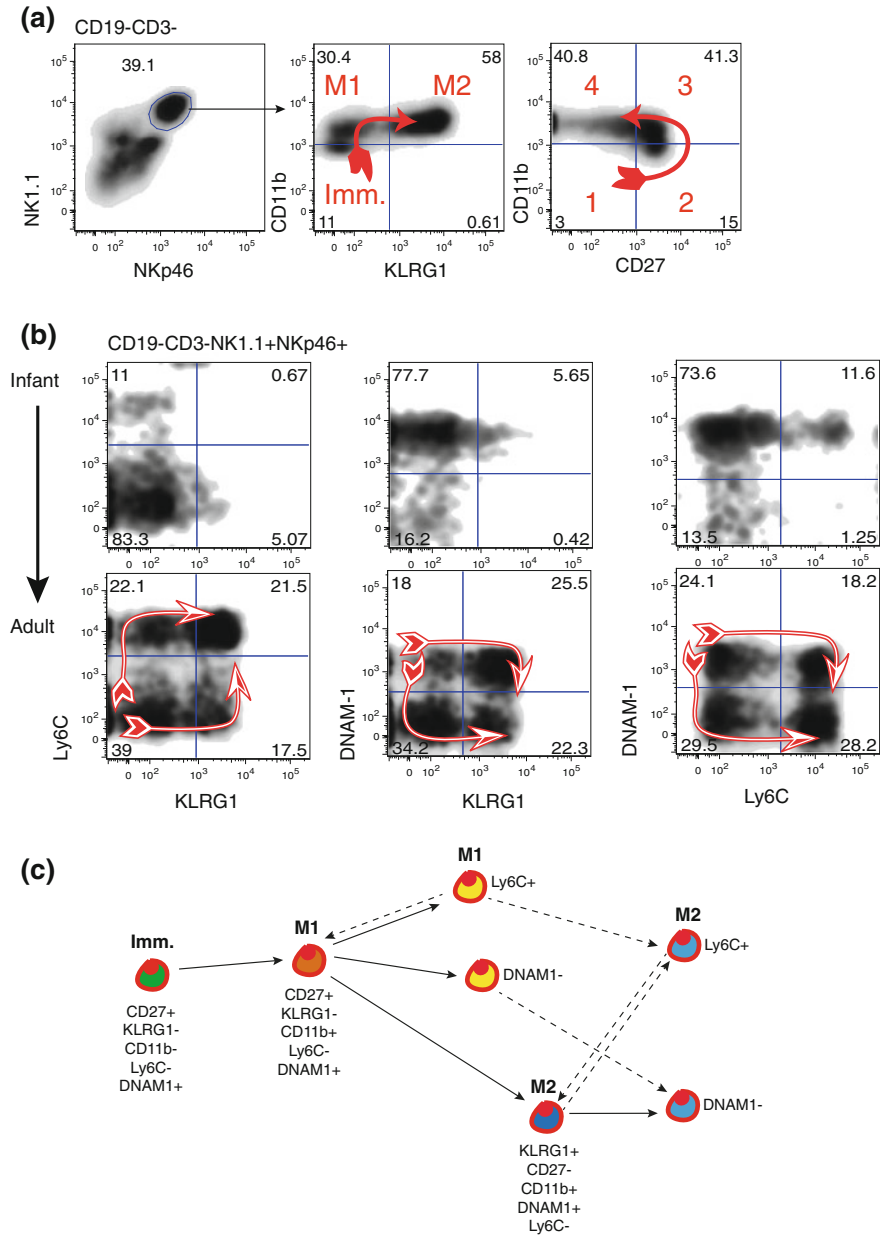
The preceding section highlights that specific transcription factor dependency controls the heterogeneity among the IFN- γ -producing ILC1 family. Among the pool cNK cells, some degree of heterogeneity also exists. NK cells are divided into at least three populations of developmentally related subsets or stages that contribute to the peripheral NK cell pool in adult mice (Hayakawa and Smyth 2006; Huntington et al. 2007b; Kim et al. 2002). These subsets differentially express a

number of surface molecules including CD43, CD11b (Mac-1), CD27, KLRG1, and the C-type lectin Ly49 multigene family of receptors (A/C/I/D/H/G2). They also display strikingly different proliferative responses to IL-15 and vary in the potency of their effector functions. These three NK cell stages were defined as (i) Immature (Imm; CD11b⁻CD27⁺KLRG1⁻), (ii) Mature 1 (M1; CD11b⁺CD27⁺KLRG1⁻), and (iii) Mature 2 or Terminal (M2; CD11b⁻CD27⁻KLRG1⁺) cells. Soon after this discovery, the group of Vivier built on the earlier discovery of the NK cell-related molecule (NKp46) in humans (Sivori et al. 1997), finding that NKp46 was highly specific to murine NK cells and conserved across multiple mouse strains. To take advantage of this finding, they generated NKp46-EGFP mice to track NK cells in vivo and proposed a 4-step NK cell (NKp46-EGFP⁺NK1.1⁺CD122⁺) maturation program (stages 1–4) in which stage 1 NK cells (CD11b⁻CD27⁻KLRG1⁻) preceded the already defined stages (Chiossone et al. 2009; Walzer et al. 2007a) (Fig. 2a). As depicted in Fig. 2a, stage 1 NK cells represent only a small fraction (1–3 %) of the splenic NK cells and were originally overlooked, however, using the NKp46 reporter mouse it has been shown that stage 1 NK cells could give rise to all other NK cell developmental stages in vivo. Building on this observation, it was found that these were also the first NK cell stage to reappear after NKp46⁺ cell depletion following induced deletion of NKp46⁺ cells in *Ncr1*-DTR mice (Chiossone et al. 2009). However, these cells exhibited some unexpected features compared with lineage-related cells. Firstly, they lacked CD27 expression, whereas all earlier NK cell progenitors in the bone marrow (NKP, rNKP, and pre-pro NK cells) and their progeny (Immature/stage 2) express CD27 (Carotta et al. 2011; Fathman et al. 2011; Klose et al. 2014). Secondly, while Immature/stage 2 NK cells were enriched in the bone marrow, stage 1 NK cells are present at the same frequency in both bone marrow and spleen. No genomic analysis of stage 1 NK cells has been performed to date but they resemble immature NK cells in terms of their expression of IL7R α (CD127) and lack of CD43 expression.

As outlined above NK cell heterogeneity clearly results from lineage differentiation, however, once NK cells reach the mature state, differences in effector functions and tissue localization may further influence their heterogeneity. This heterogeneity is imparted by a number of key surface antigens whose differential expression dissects mature NK cell effector functions and are discussed below.

3.1 *Lymphocyte Antigen 6 Complex (Ly6c)*

The glycoprotein Ly6c is commonly used to identify different monocyte populations, but is also expressed on mature NK cells (Omi et al. 2014). Ly6c⁺ NK cells first appear at the M1/stage 3 and are enriched within the M2/stage 4. It has recently been reported that Ly6c⁺ were functionally inferior to Ly6c⁻ NK cells in terms of their proliferative response to IL-15 and IFN- γ response to IL-12/18 stimulation (Omi et al. 2014). In this study, total Ly6c⁺ NK cells and Ly6c⁻ NK cells were



◀ **Fig. 2** cNK cell differentiation pathways and heterogeneity. **a** The linear differentiation of splenic cNK cells (CD19-CD3-NKp46+NK1.1+) has been defined based on CD11b, CD27, and KLRG1 expression. Solid red arrows indicate two established maturational pathways. **b** Differentiation states of NK cells from Infant (two-week old) and Adult (ten-week old) mice are shown in terms of KLRG1, Ly6c, and DNAM-1 expression. DNAM-1 and Ly6c expression are lost and gained, respectively, as NK cells differentiate to the M2 stage (acquisition of KLRG1). Arrows indicate two differentiation pathways may exist; however, this remains untested to date. **c** Schematic of the pathways giving rise to peripheral cNK cell heterogeneity. Solid arrows represent established steps while dashed arrows are proposed steps in differentiation

compared irrespective of maturation stage and did not take into account the uneven distribution of Ly6c across NK cell subsets (M1 vs. M2; Fig. 2b). Indeed, it has been found that Ly6c⁻ NK cells in fact give rise to Ly6c⁺ NK cells on adoptive transfer into recipient mice suggesting Ly6c is acquired with maturation (Omi et al. 2014). Interestingly, some plasticity in Ly6c expression exists as Ly6c⁺ cells could also revert to Ly6c⁻ following inflammation induction by polyI:C in vivo (Fig. 2c).

3.2 DNAX Accessory Molecule-1 (DNAM-1)

One of the most insightful studies into peripheral NK cell heterogeneity of late is the report from Martinet and Smyth describing the role of DNAM-1 expression in NK cells (Martinet et al. 2015). DNAM-1 (CD226) is an cell surface glycoprotein that functions as an adhesion molecule to synergizes with activating receptors and trigger NK cytotoxicity upon interaction with its ligands CD155 and CD112 (Shibuya et al. 1996). DNAM-1 is expressed by all NK cell progenitors giving rise to both DNAM-1⁺ and DNAM-1⁻ NK cells. Unlike many surface antigens characterized on NK cells, DNAM-1 does not appear to be strictly linked to maturation (Martinet et al. 2015; Nabekura et al. 2014). KLRG1⁺ and KLRG1⁻ NK cell populations contain similar fractions of DNAM-1⁺ and DNAM-1⁻ NK cells (Fig. 2b). DNAM-1⁺ NK cells produce significantly more IFN- γ , IL-6, CCL5, GM-CSF but less MIP1 α/β than their DNAM-1⁻ counterparts in response to IL-12 and IL-18. Importantly, the superior production of pro-inflammatory cytokines by DNAM-1⁺ NK cells is conserved during maturation (i.e., M1 DNAM-1⁺ > M1 DNAM-1⁻ NK cells and M2 DNAM-1⁺ > M2 DNAM-1⁻ NK cells) suggesting that loss of DNAM-1 corresponds to a functional differentiation distinct from maturation. DNAM-1⁺ NK cells also possess enhanced anti-tumor killing in vivo compared with DNAM-1⁻ NK cells although the tumor cells tested did express ligands for DNAM-1. These ligands themselves did not appear to play a role in programming these effector responses as CD155^{-/-}, CD96^{-/-}, and Tigrit^{-/-} DNAM-1 subsets retain their differential functionality. However, the group of Lanier observed reduced Ly49H⁺ NK cell expansion to murine cytomegalovirus (MCMV) infection when anti-DNAM-1 blocking antibody was injected in mice early (day 1 and 3 of infection) and in DNAM-1-null NK cells compared with control NK cells

suggesting a DNAM-1-ligand interactions can promote NK cell division or survival in vivo (Nabekura et al. 2014). Another conserved property of DNAM-1⁺ NK cells is their heightened sensitivity to IL-15 and IL-2. Responsiveness to IL-15 had previously been shown to wane with maturation and while this holds true in the study by Martinet et al. (2015), DNAM-1-expressing NK cells retained greater IL-15 sensitivity compared with their DNAM-1⁻ counterparts of similar maturation when measured by their JAK1 phosphorylation and proliferation (Martinet et al. 2015). Interestingly, DNAM-1 is uniformly expressed by liver ILC1 and at a higher level compared with DNAM-1⁺ NK cells. The functional relevance of this finding has yet to be examined.

3.3 *CD160 Antigen*

CD160 is an Ig-like glycosylphosphatidylinositol-anchored protein that resembles the killer cell Ig-like receptors and is selectively expressed on conventional NK cells with an activated or enhanced functional capacity (Anumanthan et al. 1998). NK cells in steady state are largely devoid of CD160 expression with its expression being induced by cytokines such as IL-2 and upon tumor challenge in vivo (Tu et al. 2015). CD160 deficiency results in impaired IFN- γ production by NK cells and enhanced growth of NK-sensitive tumors in vivo. As is the case with DNAM1, when CD160⁺ NK cells were sorted and assayed for IFN- γ production, they were superior to CD160⁻ NK cells and when mice were treated with CD160-Ig, tumor clearance and IFN- γ production were severely compromised (Tu et al. 2015). This indicates that acquisition of CD160 and interaction with its ligands are important steps in NK cell-mediated immune responses. Intriguingly, tissue location appears to regulate CD160 expression as all NK cells in the small intestine express high levels of CD160 (Klose et al. 2014). Furthermore, all ILC1 in both the liver and the small intestine express uniformly high levels of CD160 (Table 1).

4 Factors Regulating ILC1 and NK Cell Homeostasis

4.1 *Intrinsic Factors*

The continual expression of recombination activation genes (RAG1/2) by B- and T cells allows the generation of large pools of effector cells possessing unique antigens receptors providing broad reactivity but low affinity to protein antigens. The ability of a select few B- and T cells to recognize an antigen, improve the affinity of their antigen receptors and clonally expand to produce an army of short-lived effector or long-lived memory cells is central to our protection from an endless range of pathogens. In contrast, a relatively heterogeneous pool of short-lived NK

cells capable of secreting pro-inflammatory cytokines and cytotoxic granules exists and is the mediator of inflammation. Thus, homeostasis and activation of NK cells must also be tightly regulated in an antigen-independent manner.

4.1.1 Protein Tyrosine Phosphatase Receptor Type C—CD45

Ten years ago, we reported that the protein tyrosine phosphatase CD45 (encoded by *Ptpnrc*) is a key negative regulator of both NK cell and liver ILC1 homeostasis (Huntington et al. 2005). Hepatic ILC1 and all conventional NK cell numbers are significantly elevated in *CD45*^{-/-} mice and this correlates with increased proliferation and BrdU uptake in vivo. At the time, this observation was not surprising since our group simply referred to ILC1 as liver CD49b⁻ NK cells, thus their homeostasis was likely to be regulated in the same manner as all NK cells. However, in light of the data that ILC1 and NK cells development occurs at different stages in ontogeny and requires different transcription factors, the coevolutional control of their homeostasis (IL-15 and CD45) is intriguing and warrants further investigation. The exact mechanism of how CD45 negatively regulates NK cell and ILC1 homeostasis is not clear. CD45 has extensively been studied for its role in activating Src-family kinases required for T- and B cell antigen receptor signaling and relevant to this review, we previously described a role for CD45 in signaling cytokine production downstream of ITAM-containing receptors on NK cells. Interestingly, the group of Penninger (Irie-Sasaki et al. 2001) proposed that CD45 can act as a negative regulator of IL-3, IFN- α , IL-4, and EPO signaling by dephosphorylating JAK1/2. In contrast, others failed to observe cytokine hyper-responsiveness to IL-4 on *CD45*^{-/-} B cells in vitro (Huntington et al. 2006). As discussed in detail throughout this review, ILC1 and NK cells are dependent on IL-15 with this cytokine playing an essential role in NK cell differentiation. *CD45*^{-/-} mice have block in T cell development in the thymus and subsequently present with peripheral T lymphopenia. Consistent with other T lymphopenic strains, *Rag1*^{-/-} and *Tcra*^{-/-} (Kamimura and Lanier 2015), around 70–90 % of peripheral NK cells, are KLRG1⁺ in *CD45*^{-/-} mice as opposed to 30–50 % in T cell sufficient control mice. However, while ~80 % of the *CD45*^{-/-} NK cells remained KLRG1⁺ in mixed bone marrow chimeras, only ~30 % of the WT NK cells were KLRG1⁺ indicating that CD45 plays a cell intrinsic role in NK cell homeostasis and differentiation.

4.1.2 Ikaros Family Members Aiolo and Helios

While most research on transcription factors in the NK cell field focus on their role in NK cell versus ILC commitment from progenitors, several groups including our own have addressed their role in the differentiation of peripheral NK cell subsets and functional diversification. The Ikaros family of zinc finger proteins Aiolo (encoded by *Ikzf3*) is expressed early during hematopoiesis and regulates aspects of

lymphoid lineage development (Morgan et al. 1997). *Irf3* is highly expressed in NK cells from the pre-pro NK cell stage onwards and *Irf3*^{-/-} mice display an unusual arrest in NK cell differentiation at the transition from M1 to M2 stage (Holmes et al. 2014). As a result, most peripheral NK cells in *Irf3*^{-/-} mice fail to down-regulate CD27 and are Mac1⁺CD27⁺KLRG1⁺. In addition, they unexpectedly express c-kit and CD51. *Irf3*^{-/-} peripheral NK cells are more sensitive to the homeostatic cytokines IL-2 and IL-15 most likely as a result of failing to fully terminally differentiate. Unexpectedly, *Irf3*^{-/-} NK cells produce less IFN- γ but displayed superior in vivo killing of NK cell sensitive tumors, some of which are highly sensitive to IFN- γ . The mechanisms behind such a dichotomy remain unclear. Transcription factors known to regulate NK cell development were normally expressed in *Irf3*^{-/-} NK cells; however, Helios (*Irf2*) was significantly up-regulated. *Irf2* is up-regulated in immature NK cells before being silenced in mature NK cells, possibly by signals mediated through NKp46. *Irf2* expression has been associated with functionally superior or hyperactive NK cells which could explain the enhanced tumor killing in *Irf3*^{-/-} mice (Narni-Mancinelli et al. 2013).

4.1.3 B-Lymphocyte-Induced Maturation Protein-1 (Blimp-1)

The arrested NK cell differentiation phenotype of *Irf3*^{-/-} mice resembles that of B-lymphocyte-induced maturation protein-1 (Blimp-1, encoded by *Prdm1*) deficiency (Holmes et al. 2014; Kallies et al. 2011). Blimp-1 is a zinc finger containing transcription factor that plays a key role in the terminal differentiation of B- and T cells. Blimp-1 expression increases during NK cell differentiation and is rapidly up-regulated upon IL-12 and IL-21 stimulation. Deletion of Blimp-1 results in an NK cell intrinsic failure to transit from the M1 to M2 stage and subsequently the Blimp-1^{gfp/gfp} (knock-in allele) NK cells have very few KLRG1⁺ NK cells and a reduction in Mac1⁺ NK cells. This does not appear to influence effector function as Blimp-1^{gfp/gfp} NK cells are competent cytokine and cytotoxic granule producers in vitro (Kallies et al. 2011). In line with their similar NK cell phenotype to *Irf3*^{-/-} mice, Blimp-1^{gfp/gfp} mice also display superior in vivo tumor killing and proliferative response to IL-15. These data support the notion that M1 NK cells are a key cytotoxic population within the innate lymphocyte lineage with potent anti-tumor responses and heightened sensitivity to inflammatory cytokines such as IL-15.

4.1.4 Forkhead Box Protein O1 (Foxo1)

Forkhead box protein O1 (Foxo1) is a transcription factor of the forkhead family that was recently identified as a negative regulator of NK cell differentiation. While relatively mild compared to *CD45*^{-/-} mice, *Foxo1*^{fl/fl}*Ncr1-iCre* mice present an increased M2 population of NK cells in the periphery and reduced numbers of NK cells in lymph nodes owing to a down-regulation of CD62L among the Imm. fraction of NKp46⁺ NK cells (Deng et al. 2015). Similarly to Foxo3a (Huntington et al. 2007a),

the homeostatic cytokines IL-2 and IL-15 induced the phosphorylation of Foxo1, preventing it from binding to its target genes. One target gene analyzed in this study was T-bet. T-bet is essential for NK cell differentiation and *Tbx21*^{-/-} mice do not develop M2 NK cells and have significantly fewer total NK cells (Daussy et al. 2014; Gordon et al. 2012). The expression of T-bet and Foxo1 during NK cell ontogeny is inversely with T-bet increasing and Foxo1 decreasing during differentiation. FOXO1 was found to bind Tbx21 at the proximal promoter region and Tbx21 expression was significantly elevated in *Foxo1*-null NK cells indicating that FOXO1 acts as a repressor of T-bet expression to limit NK cell differentiation in vivo. Indeed, the overrepresentation of M2 NK cells in *Foxo1*^{fl/fl}*Ncr1-iCre* was abolished when *Tbx21* was also deleted supporting the proposed role of FOXO1 acting via T-bet (Deng et al. 2015).

4.2 Extrinsic Factors

The current understanding of lymphocyte homeostasis is that when a system experiences a deficiency in a cell type due to infection/chemotherapy/irradiation, the biological system will induce a replenishment of these cells from progenitors or residual cells. For example, T cells proliferate rapidly to repopulate a T cell deficient host. Whether the same rules apply to NK cells is unclear. The principle factor known to regulate NK cell homeostasis is IL-15 but IL-15 is also necessary for the development and maintenance of other lymphocyte subsets. Thus, a key question is how competition for IL-15 by IL-15-dependent lymphocytes is orchestrated to regulate their homeostasis.

4.2.1 Interleukin-15 (IL-15)

IL-15 is the key cytokine required for NK cell homeostasis and has been reviewed extensively of late (Huntington 2014; Waldmann 2014). The competition for IL-15 in NK cell homeostasis was recently examined by the group of Lanier in the context of anti-MCMV responses (Kamimura and Lanier 2015). Consistent with earlier studies, this group observed that IL-15 was important for driving NK cell maturation and specifically the up-regulation of KLRG1 (Huntington et al. 2007b). They build on this finding by arguing that T cells compete for IL-15 in steady-state settings as mice lacking T cells (*Rag1*^{-/-} or *Tcr α* ^{-/-}) had a peripheral NK cell pool with significantly more KLRG1⁺ NK cells. Furthermore, the fraction of KLRG1⁺ NK cells in *Rag1*^{-/-} mice could be reduced to normal levels by the restoration of T cells into the system either via injection of total splenocytes into *Rag1*^{-/-} mice or by the generation wild type: *Rag1*^{-/-} mixed bone marrow chimeras. We have previously shown that homeostatic proliferation of NK cells results in the accumulation of terminally mature KLRG1⁺ NK cells (Huntington et al. 2007b). This group reasoned that IL-15 was critical for the enhanced homeostatic proliferation and

accumulation of KLRG1⁺ NK cells in *Rag1*^{-/-} mice as deletion of one or both copies of *Il15* resulted in a dose-dependent reduction in KLRG1⁺ NK cells. They also propose a role for commensal bacteria in the homeostatic expansion of NK cells as antibiotic treatment reduced the fraction of KLRG1⁺ NK cells in vivo and *Nod1/2*^{-/-} possessed fewer KLRG1⁺ NK cells than wild-type counterparts. This is potentially also linked to IL-15 production via myeloid and non-hematopoietic cells as a result of NOD signaling. This competition between NK cells and T cells for IL-15 and commensal bacteria has important consequences for immune responses. In response to MCMV, KLRG1⁻ NK cells are functionally superior to KLRG1⁺ NK cells and experience a significantly greater Ly49H-m157 expansion 7 days of post-infection. This finding confirms our early work demonstrating that KLRG1⁺ NK cells have a very poor proliferative capacity in response to IL-15 in vitro and following transfer into immunodeficient *Rag2*^{-/-} γ *C*^{-/-} mice (Huntington et al. 2007b). Taken together, these data reiterate the importance of extrinsic factors regulating NK cell homeostasis via IL-15; however, others have argued an intrinsic role for Rag in regulating this process. Karo et al. (2014) recently presented similar experimental evidence to that of the Lanier group (Kamimura and Lanier 2015) showing Rag-deficient NK cells are inferior to wild-type NK cells in terms of Ly49H expansion in responses to MCMV (Karo et al. 2014). However, their conclusions were quite different arguing that Rag-deficient NK cells possessed a cell intrinsic hyper-responsiveness phenotype associated with enhanced apoptosis resulting from greater genome instability/reduced DNA break repair. While a direct, cell intrinsic role for *Rag1/2* in regulating NK cell homeostasis remains possible, it is clear that an absence of T cells and excessive IL-15 can alter the homeostasis of NK cells which can influence the magnitude of NK cell anti-viral immunity.

Table 1 Phenotype of NK cells and tissue-resident ILC1

| | NK1.1 | NKp46 | CD49a | CD49b | CXCR6 | IL7R | TRAIL | CD160 | CD226 | Ly49E | CD11b | KLRG1 | CD62L |
|-------------|-------|-------|-------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|
| NK cells | | | | | | | | | | | | | |
| Liver ILC1 | | | | | | | | | | | | | |
| Thymic ILC1 | | | ND | | ND | | | ND | ND | ND | | | ND |
| I.E ILC1 | | | | | | | | | | | | | |



4.2.2 Interleukin-2 (IL-2)

IL-15 and IL-2 are both members of the γ_C cytokine family and have overlapping signaling pathways (JAK1/3 and STAT5) and overlapping functions (NK cell activation and proliferation). Furthermore, the structures of the IL-15 and IL-2 signaling complexes look very similar yet *IL-2*^{-/-} and *IL-15*^{-/-} mice present very different phenotypes suggesting unique roles for these cytokines in NK cell homeostasis (Kennedy et al. 2000; Ring et al. 2012; Schorle et al. 1991). Both IL-2 and IL-15 require the heterodimeric IL-2R β / γ complex for signaling and a third cytokine-specific α chain can enhance signaling and affinity in the case for IL-2 (IL-2R α /CD25), whereas IL-15R α is required to trans-present IL-15 to IL-2R β / γ expressing cells but does not intrinsically alter IL-15 signaling (Lodolce et al. 1998). The group of Rudensky recently investigated the competition for IL-2 between regulatory T cells (Treg) and NK cells in vivo (Gasteiger et al. 2013a, b). On deletion of Treg (which express high affinity IL-2 receptors and are highly dependent on IL-2 for homeostasis), a significant increase in immature CD127⁺CD25⁺ NK cells was observed. Consistent with earlier studies, these immature NK cells appear highly sensitive to IL-2R β / γ agonists and interestingly, their function was also enhanced following Treg depletion suggesting that competition for IL-2 during infection may shape the magnitude of NK cell immunity (Huntington et al. 2007b; Gasteiger et al. 2013a, b).

4.2.3 Transforming Growth Factor Beta (TGF- β)

Transforming growth factor beta (TGF- β) is a potent immune-regulatory cytokines. TGF- β 1 is the predominant isoform produced by a large range of cell types including most hematopoietic cells and is associated with a latent complex and must be release prior to binding its receptors. TGF- β signaling via TGF-RI and RII complexes involves phosphorylation of the receptor-associated SMAD molecules and has a well-documented inhibitory effect on NK cell function and development (Li et al. 2006). Flavel and colleagues reported a 5–10 fold expansion in NK cell frequency in mice expressing a dominant-negative form of the TGF- β RII driven by the CD11c promoter (CD11c-dnTGF β RII) (Laouar et al. 2005). NK cells from CD11c-dnTGF β RII failed to induce SMAD2 phosphorylation following TGF- β 1 stimulation and were resistant to the inhibitory effects of TGF- β 1 in terms of IFN- γ production. As a consequence, CD11c-dnTGF β RII mice generated more potent immune responses to *Leishmania Major* and MCMV, with greater IFN- γ and NK cell expansion compared to control aged match mice NK cells (Laouar et al. 2005; Marcocoe et al. 2012). Treatment of neonatal mice with TGF- β 1 substantially impairs NK cell development and maturation and given the opposite phenotype is seen in CD11c-dnTGF β RII mice suggests that TGF- β 1 is produced during normal, healthy hematopoiesis to regulate the degree of NK cell homeostatic expansion and activation.

Dendritic cells are a possible source of TGF- β 1 for NK cells during immune responses and recently dendritic cells activation via STAT3 was shown to suppress NK cell activity in part by altering TGF- β 1 and IL-12 levels (Sarhan et al. 2015).

5 Conclusions and Perspectives

- The ILC field research is rapidly expanding and with this we have identified substantial heterogeneity within ILCs.
- The development of highly specific genetic mouse models with ILC deficiencies is required to accurately delineate the unique and shared functions of ILC subsets.
- NK cells have long been considered a single ILC1 subset. Given the newly emerging data, ILC1 are a distinct class of innate cells, necessitating reassessment of both NK cell and ILC unique contributions to immunity.
- Tissue location appears to be a key factor in driving phenotypic heterogeneity. This suggests unique roles for distinct tissue-resident NK cell and ILC1 subsets.
- Phenotypically we can identify anywhere from 3 to 10 NK cell subsets based on markers discussed in this review, the question that needs addressing is the stability or plasticity within these subsets and the evolutionary role of generating such diversity.

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