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ID'ing Innate and Innate-like Lymphoid Cells

Mihalis Verykokakis, Erin C. Zook, and Barbara L. Kee

Committee on Immunology and Department of Pathology, The University of Chicago, Chicago IL, USA

Summary

The immune system can be divided into innate and adaptive components that differ in their rate and mode of cellular activation, with innate immune cells being the first responders to invading pathogens. Recent advances in the identification and characterization of innate lymphoid cells have revealed reiterative developmental programs that result in cells with effector fates that parallel those of adaptive lymphoid cells and are tailored to effectively eliminate a broad spectrum of pathogenic challenges. However, activation of these cells can also be associated with pathologies such as autoimmune disease. One major distinction between innate and adaptive immune system cells is the constitutive expression of ID proteins in the former and inducible expression in the latter. ID proteins function as antagonists of the E protein transcription factors that play critical roles in lymphoid specification as well as B and T-lymphocyte development. In this review, we examine the transcriptional mechanisms controlling the development of innate lymphocytes, including natural killer cells and the recently identified innate lymphoid cells (ILC1, ILC2, and ILC3), and innate-like lymphocytes, including natural killer T cells, with an emphasis on the known requirements for the ID proteins.

Keywords

natural killer cells; natural killer T cells; T-helper cells; transcription factors; cell differentiation

Introduction

The immune system is a complex cellular network that needs to be tightly coordinated and appropriately regulated to protect against pathogens, autoimmune disease, and cancer. This system is divided into adaptive and innate components (1). Adaptive immune cells express antigen-specific surface receptors that are generated by genomic recombination of gene segments whose combinatorial diversity allows for greater than 10^{10} unique specificities. While the diversity of antigen receptors expressed by adaptive immune cells is enormous, each cell is unique and requires proliferative expansion to mount an effective response to a pathogen. Because clonal expansion is required, adaptive immune responses peak 7 to 14 days after recognition of an invading pathogen. In addition, adaptive immune cells are maintained in a naive effector state and must be primed/activated during the course of clonal

Correspondence to: Barbara L. Kee, Department of Pathology, The University of Chicago, 927 E 57th St., JFK Rm 318, Chicago, IL 60637, Tel.: +1 773 702 4349, Fax: +1 xxxxxx, bkee@bsd.uchicago.edu.

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expansion. Therefore, immediate control of a pathogenic infection requires innate immune cells that rely on germ line-encoded receptors to provide a fast, albeit largely non-pathogen specific, response. Innate immune cells can influence the ensuing adaptive immune response through multiple mechanisms including through the production of cytokines, which prime and direct adaptive cells, and by controlling the amount of pathogen available to activate the adaptive immune cells. Adaptive and innate immune cells develop in the fetal liver and adult bone marrow from hematopoietic stem cells (HSCs) that also give rise to the cells required for erythropoiesis and hemostasis (2). The vast majority of innate cells derive from myeloid precursors, whereas the adaptive immune cells arise from a common lymphoid progenitor (CLP) (3).

Despite the functional and developmental segregation of many innate and adaptive immune cells, there are cells that cross these boundaries. Natural killer (NK) cells are lymphocytes and develop from CLPs but they are innate immune cells that recognize pathogen using germ line encoded receptors and they exist in a primed state. The activity of NK cells was first documented more than 40 years ago and these cells were the only known innate lymphoid cell subset until very recently. However, numerous recent studies identified several additional innate lymphoid cell subsets, primarily IL7R α ⁺ cells called ILCs, whose functional and developmental relationship to NK cells is emerging (4, 5). In this review, we use the acronym ILC for these newly identified IL7R α ⁺ innate lymphoid cells. In addition to NK cells and ILCs, T and B-cell populations have been discovered that share characteristics with innate immune cells and are thus named innate-like T and B cells. Among them, Natural killer T (NKT) cells, some $\gamma\delta$ T cells, and marginal zone B cells (MZB) are examples of lymphocytes that express rearranged antigen receptors with limited diversity and have the ability to produce large amounts of cytokines rapidly after stimulation (6–8).

Over the past year, striking advances have been made in our understanding of the programs controlling the development and function of innate lymphoid cells and NKT cells (9–11). Foremost among these is the finding that these programs share a common developmental and effector structure with the conventional T-lymphocyte program (Fig. 1). The notion that NK cells represent an innate counterpart to CD8⁺ T cells has been raised previously, and NK cells share a core effector program with activated CD8⁺ T cells (12, 13). However, ILCs and NKT cells acquire effector functions that closely resemble the CD4⁺ T-helper (Th) cell programs. The Th1, Th2, and Th17 programs share properties with the ILC1, ILC2, ILC3 and the NKT1, NKT2, and NKT17 programs, respectively (Fig. 1). These effector programs are defined by the expression of signature transcription factors that are required for their development and the cytokines that they produce: TBET and IFN γ define Th1/ILC1/NKT1 cells, GATA3 and IL4/IL5/IL13 define Th2/ILC2/NKT2 cells, and ROR γ t and IL17/IL22 define Th17/ILC3/NKT17 cells. Broadly, the Th1-related programs are activated in response to intracellular pathogens such as viruses, the Th2 related programs are activated in response to helminthes and epithelial damage and the Th17-related programs are activated by bacterial infection and inappropriate activation of any of these pathways contributes to disease. A major challenge for the future is to uncover the molecular mechanisms that determine the representation of each lineage downstream of their common progenitors. For the NKT lineage, different strains of mice have a unique representation of these programs in the thymus, suggesting that program choice is genetically determined (11, 14). Moreover,

the absolute number of NKT2 cells can influence many aspects of the immune response and when NKT2 cells are at their highest, as in Balb/c mice, they may skew the immune bias toward a Th2 response (11). Whether overlapping or distinct molecular mechanisms are used by innate and adaptive lymphoid cells to determine program choice remains to be resolved. In this review article, we examine our current understanding of the developmental pathways leading to the ILC and the NKT cell programs and the transcriptional regulatory networks that guide these processes. A major emphasis will be on the ID proteins, inhibitors of E protein transcription factor activity, whose constitutive expression in innate lymphoid cells distinguishes them from their adaptive counterparts.

ID and E proteins

The Inhibitor of DNA-binding (ID) proteins are transcriptional regulators that belong to the Helix-Loop-Helix (HLH) protein family and are central mediators of developmental processes across species ranging from flies to mammals. In *Drosophila melanogaster*, there is a single ID-like protein, encoded by the *extra macrochaete (emc)* gene that functions in sensory organ development, whereas mammals contain four ID members (ID1-4) that control cell fate decisions throughout development (15). ID proteins exert their functions through heterodimerization with Class I basic HLH (bHLH) family of transcription factors, which are also called E proteins. Because ID proteins lack the basic region that mediates DNA binding, such interactions prevent E proteins from associating with their cognate DNA elements, thus inhibiting their transcriptional activity. In mammals, there are 3 E protein genes that encode 6 highly homologous bHLH proteins that bind DNA sequences called E boxes. The different E proteins arise through alternative transcriptional initiation or differential splicing of the *Tcf3* (encoding for E12 and E47, known as E2A), *Tcf4* (E2-2can and E2-2alt) and *Tcf12* (HEBcan and HEBalt) genes. E proteins contain two autonomous transcriptional activation domains (AD1 and AD2) at their N-terminus and, upon bHLH-mediated homodimerisation, they act mainly as transcriptional activators through recruitment of co-activators such as p300/CBP and histone acetyl-transferases. E proteins can also dimerize with class II bHLH proteins resulting in either transcriptional activation or repression depending on the dimerizing partner and the cellular context (16).

ID and E proteins are widely expressed throughout hematopoietic cells and biochemical and genetic analysis suggests that their relative abundance determines the final E protein transcriptional outcome (17). The E proteins are essential for B and T-cell development and regulate genes involved in lymphoid lineage specification, commitment and antigen receptor gene rearrangement (18–22). In contrast, ID3 is induced downstream of T-cell receptor (TCR and pre-TCR) signaling, thus enforcing the β -selection and positive selection checkpoints by extinguishing antigen receptor recombination and inhibiting differentiation stage-associated genes (23, 24). Mice deficient in E2A have few T lymphocytes, but they succumb to T-cell lymphomas with an immature phenotype, whereas HEB is critical in later stages of T-cell development to control DP survival and TCR α recombination (25–27). *Id3*^{-/-} mice have an apparent failure of positive and negative selection and develop autoimmune disease as well as $\gamma\delta$ T-cell lymphomas (28–30). In humans, T and B-lymphocyte lineage acute lymphoblastic leukemia cells frequently have mutations that affect E protein activity and B-cell lymphomas are characterized by loss-of-function ID3 mutations

and/or gain-of-function E2A mutations (31–33). Therefore, tight regulation of the ID/E protein pathway is essential not only for proper lymphocyte development but also to prevent lymphoid malignancy.

In contrast to adaptive lymphoid cells, innate lymphoid cells express ID proteins constitutively. ID2-deficient mice were initially reported to lack mature NK cells and secondary lymphoid tissues due to a failure to generate lymphoid tissue-inducer (LTi) cells, a cell type that is now known to be a member of the ILC3 family (34). This observation, and the knowledge that B lymphocytes and NK cells develop from CLPs, led to the hypothesis that ID2 promotes NK cell/LTi cell development by inhibiting E protein dependent B and T-lymphocyte lineage specification and commitment. The recently identified ILC1, ILC2, and ILC3 all highly express ID2 and require ID2 for their development leading to the hypothesis that these cells, along with NK cells arise from a common innate lymphoid progenitor (35, 36). However, it was shown many years ago that NK cell lineage specification is not dependent on ID2, likely due to compensation by ID3 (37), whereas ILC development appears to be highly ID2 dependent. Consistent with these studies, an ID2-expressing progenitor for all ILCs, but distinct from NK cell progenitors was recently identified (10). Therefore, the requirements for ID2 may initiate in different precursors giving rise to ILCs and NK cells; however, to date there has been no clear insight into why ID proteins are required for development of innate lymphoid cells. Recent studies into the role of ID proteins in NKT cells may help to provide insight into this question, as discussed below.

Innate lymphoid cells

Natural killer cells

Innate lymphoid cells have been divided into three groups based on their functional properties (38). NK cells are included in Group 1, and in many respects, their development, gene expression profile, and function places them as an innate counterpart to CD8⁺ T cells (13). Similar to CD8 T cells, NK cells kill virus infected and transformed cells through the release of granzymes and perforin and they secrete copious amounts of inflammatory cytokines such as IFN γ and TNF α . The mechanisms controlling NK cell development have been investigated primarily in the bone marrow, although immature NK cells can be detected in many peripheral lymphoid tissues as well as in the liver. The identity of the progenitors downstream of CLPs that give rise to mature NK cells are not well established and their phenotype overlaps significantly with the progenitors of ILCs. CLPb are Lin[−]CD127⁺*ckit*^{int}Sca1^{int}Flt3[−] cells that give rise to B lymphocytes and macrophages *in vitro* but have extinguished T-lymphocyte potential, and these properties correlate with increased expression of *Id2* compared with CLPs and a lack of *Notch1* transcription (39). *Notch1* is a known target of E proteins that is expressed in multipotent progenitors, and deletion of *Id2* and one allele of *Id3* was sufficient to restore *Notch1* expression and T cell potential in CLPb. Therefore, CLPb have a more restricted developmental potential than CLP and the mild but functionally relevant upregulation of *Id2* is sufficient to restrict T-cell potential directing these cells closer to the NK cell lineage. These data are consistent with the hypothesis that ID2 restricts the lymphoid potential of progenitors downstream of CLP. However, the ability of these cells to generate ILC has not been tested, so it remains unclear

whether these cells represent a progenitor before or after the bifurcation point of the NK and ILC lineages. However, Notch signaling is required for expansion of ILC suggesting that CLPb may have lost ILC differentiation potential (40).

The pathway to mature NK cells was defined as proceeding through an NK progenitor (NKP) that was originally defined as Lin⁻CD122⁺NK1.1⁻DX5⁻ (41). In 2011, Fathman *et al.* (42) identified a bone marrow population that was Lin⁻CD122⁺Flt3⁻CD244⁺CD27⁺CD127⁺, called a revised NKP, which gave rise to NK1.1⁺ cells *in vitro* and *in vivo*. While the inclusion of CD244 and CD27 in the definition of NKPs removed T-cell contamination, it remains unclear whether the CD127⁺ subset represents a true NK progenitor or a progenitor of ILCs, all of which express CD127 unlike mature (m) NK cells. Nonetheless, cells within the NKP population upregulate NK1.1 to become immature (i) NK cells before expressing DX5 a marker of mNK cells (41, 43). However, even within the iNK cell population there are CD127⁺ cells that may be assigned to the ILC1 program. Mature NK cells can be distinguished from ILC1 by their expression of the T box transcription factor EOMESODERMIN (EOMES), whereas both NK cells and ILC1 express TBET. Interestingly, while TBET is absolutely essential for development of ILC1 (10, 44), NK cells have both a cell intrinsic and extrinsic requirement for TBET that effects NK cell maturation (45, 46). In contrast, only mNK cells require EOMES for their development (10).

While several transcription factors are known to be required for the development or maintenance of mature NK cells, to date, there have been no factors identified as being essential for NKPs. The failure to find an essential NKP transcription factor may reside in the heterogeneity within the originally defined Lin⁻CD122⁺ population, as it is composed of activated T cells, true NKP and, likely, ILC. This is an issue that should be resolved in the near future as the identity of true NKP becomes resolved. ID2, E4PB4, ETS1, EOMES, and TOX all play important roles in NK cell development (47), whereas HELIOS, TBET, BLIMP1, and ZBTB32 play a critical role in NK cell maturation or function (48–50). We will focus here on the transcription factors that regulate the development of mNK cells from CLP.

The regulation of ID2 has emerged as a critical control point for NK cell, and likely ILC, development. Deletion of *Id2* results in a reduced number of mNK cells in both the bone marrow and the spleen and a block at the iNK stage due to a failure to antagonize E protein activity (37). E4BP4-deficient mice have a reduced number of iNK and mNK cells, and E4BP4 appears to function, at least in part, by regulating *Id2* and *Eomes* (51–53). ETS1-deficient mice also have reduced numbers of NK cells and ETS1 functions, in part, to regulate expression of *Id2* (54, 55). However, ETS1 regulates many other genes in NK cells, including genes encoding NK cell receptors and critical signaling molecules, and it is required for the development of the rNKP (54), indicating that ETS1 may be required for ILC development as well. *Tox*^{-/-} mice have a phenotype remarkably similar to ID2-deficient mice; however, ectopic expression of ID2 in TOX-deficient progenitors did not restore NK development *in vitro*. Thus, TOX may have essential targets beyond ID2 that control NK and ILC development (56). In Fig. 2, we have assembled a basic transcriptional regulatory network that summarizes the known interactions between these early acting

transcription factors in NK cell development. Nonetheless, an understanding of how these factors cooperate to control the emergence of appropriately primed mature NK cells remains to be fully resolved.

ILC1

ILC1, like NK cells, are Group 1 innate lymphoid cells that likely play a key role in the detection of intracellular pathogen and priming of the adaptive immune response. ILC1 are CD127⁺NK1.1⁺ TBET expressing cells that produce IFN γ and function in the clearance of intracellular pathogens (10). Using an Eomes-GFP reporter mouse, Daussy et al were able to distinguish Eomes expressing NK cells from ILC1 in the liver (44). They demonstrated that Eomes-GFP⁻ hepatic NK1.1⁺DX5⁻Trail⁺CD11b⁺ cells (ILC1) cannot differentiate into Eomes-GFP⁺ mNK cells when adoptively transferred into mice indicating that ILC1 are a stable lineage distinct from NK cells. Using the EOMES reporter mouse they were also able to show that development of ILC1 requires TBET whereas NK cell development does not. In the mucosa of the gut Eomes⁻TBET⁺NK1.1⁺ ILC1s are also distinct from Eomes⁺ NK cells (10). ILC1s that express EOMES and TBET, but are distinct from NK cells, have been identified in the human tonsil and gastrointestinal track (57). These human ILC1 cells secrete IFN γ in response to IL12 and IL15. While IFN γ secretion is common to all ILC1s their phenotype in each organ differs slightly suggesting that there may be environmental or tissue specific phenotypes and functions of ILC1s.

In contrast to NK cells, which are largely IL15-dependent cells, ILC1 are defined by their expression of the IL7 receptor, raising the question of whether ILC1 require IL7 for their survival. Notably, in the liver and gut, the number of ILC1 is similar in *Il7ra*^{-/-} and WT mice. In contrast, and similar to NK cells, both liver and gut ILC1s are dependent on IL15 for their development (10, 44, 58, 59). To date, the transcriptional requirements for ILC1, beyond the need for TBET, have not been extensively investigated. However, their cytokine dependence places them close to the NK cell lineage in terms of developmental requirements and suggests that many of the factors required for NK cell development may also be required for ILC1.

ILC2

ILC2s play an important role in helminth expulsion and airway hyper-reactivity (36, 60–62). ILC2s lack the expression of lineage specific cell surface markers but express ICOS, CD127, CD25, CD90, SCA1, ST2 (IL33R), and IL25R. Committed ILC2 progenitors (ILC2P) that express $\alpha 4\beta 7$ have been identified in the bone marrow (9) and mature ILC2s that express KLRG1 have been identified in peripheral organs including the gut, lungs, and mesenteric lymph nodes (63). While several transcription factors are required for ILC2 development the transcriptional hierarchy and essential targets of each transcription factor remain to be fully characterized.

ROR α , GATA3, TCF-1, and GFI-1 all play a role in ILC2 development. ROR α -deficient mice have a reduced number of ILC2 and the few ILC2 that develop fail to respond to IL25 or IL33 resulting in an inability to clear the helminth *Nippostrongylus brasiliensis* (40, 60). While a role for ROR α in ILC2 development is evident, it is not known whether ROR α also

affects ILC2 survival, proliferation, and/or effector function. Additionally, while ILC2 progenitors (ILC2P) in the bone marrow express ROR α , it is not known how ROR α expression is regulated. ILC2s in the bone marrow also express GATA3 and GATA3 is critical for the development of ILC2s. GATA3 expression increases with developmental progression of CLP to ILC2P to mature ILC2s (64). ILC2s are absent in mice when GATA3 is deleted using an ID2 inducible cre (63). ILC1 and ILC3 are also dependent on GATA3 expression as a common ILC progenitor downstream of CLPs requires GATA3 for development (65, 66). Using *Il13^{Yecre}* to delete *Gata3* it was shown that GATA3 is also required for IL13 production in mature ILC2 cells (67). Thus, GATA3 is not only required for the development of ILC2s but also for their cytokine production.

TCF1- and GFI1- deficient mice have reduced numbers of ILC2s and reduced GATA3 expression, suggesting that TCF1 and GFI1 are upstream of *Gata3*. (64, 68) TCF1 expression can be induced by Notch signaling which is also required for ILC2 lineage restriction. When TCF1 is over expressed GATA3 expression increased further supporting the notion that TCF1 can regulate *Gata3*. Additionally, ChIP experiments demonstrated that TCF1 can bind to the *Gata3* promoter as well as the *Il7ra* promoter. Similarly, chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) revealed that GFI1 binds to *Gata3* as well as additional ILC2 genes including *Il1r1* (encoding IL33R), *Il17*, and *Il15* (64). Thus, GFI1 not only regulates ILC2 development by contributing to *Gata3* expression but also plays a role in ILC2 effector function. GFI1-deficient mice had reduced expression of IL5 and IL13 with increased expression in IL17 indicating that GFI1 is required for proper cytokine expression in activated ILC2s. While both TCF1 and GFI1 can bind to the *Gata3* locus it is unknown if TCF1 can regulate expression of GFI1 or vice versa.

ILC2s require IL7 signaling for their development which separates them from ILC1s and NK cells (36). ILC2s were absent in the mesentery of IL7R^{-/-} and γ c^{-/-} mice. Interestingly, ILC2 were not reduced in IL15 or IL15 receptor deficient mice (36). These findings demonstrate a clear difference in the cytokine requirements for the development of group 2 ILCs and group 1 ILCs.

ILC3

Group 3 ILC are comprised of LT α i cells, NCR⁻ ILC3s, and NCR⁺ ILC3s. Additionally, ILC3s contain an additional ILC3 subset that can convert to an ILC1-like IFN γ secreting cell. LT α i cells are required for lymph node and Peyer's patches formation in fetal and early post-natal life and for the formation of cryopatches and isolated lymphoid follicles (69). LT α i cells express CCR6 and can be subdivided into CD4⁻ LT α i0 and CD4⁺ LT α i4 cells, where LT α i0 give rise to the LT α i4 cells. NCR⁻ and NCR⁺ ILC3 are CCR6⁻ and play a critical role in protecting the gut against bacteria such as *Citrobacter rodentium* by producing IL22 that promotes the production of adhesion molecules and antimicrobial peptides in epithelial cells (70).

All members of the group 3 ILCs require expression of ROR γ t for their development. ROR γ t reporter mice, in which EGFP is inserted into the first exon of *Rorc* (resulting in a nonfunctional allele), do not contain lymph nodes, Peyer's patches, and LT α i cells indicating

that ROR γ t is essential for their development (71). NCR positive and negative ILC3s are also missing in ROR γ t-deficient mice (70, 72, 73). Several of the transcription factors mentioned in previous sections, including TOX, GATA3, and TCF1, also play a role in ILC3 development. These transcription factors may be required for the generation of a common ILC progenitor; however, it cannot be ruled out that some of these transcription factors are required at different stages of development in distinct ILC lineages, in addition to being required for ILC effector function. *Tox*^{-/-} mice do not have lymph nodes and have reduced frequency and size of Peyer's patches indicating a role for TOX in LT α i development (56). TOX is also required for NK cells however a role for TOX in ILC1s and ILC2s remains to be demonstrated. Similar to ILC2s, TCF1 and GATA3 are required for the generation of ILC3s (65, 74). TCF1 is intrinsically required for the development of NCR⁺ ILC3s but not NCR⁻ or LT α i cells. On the other hand, GATA3 is required not only for the generation of NCR⁺ ILC3 but also for CD4⁺ ILC3 LT α i cells. Mice deficient in GATA3 have higher bacterial loads when infected with *C. rodentium* due to a decrease in the IL22 producing ILC3s. Aryl hydrocarbon (AHR)-deficient mice also fail to control *C. rodentium* due to a requirement for AHR in the development and function of adult ILC3. In contrast, AHR is not critical for development of fetal ILC3s and therefore *Ahr*^{-/-} mice have Peyer's patches and lymph nodes (75–77)

In both mice and humans, it was demonstrated that a subpopulation of ILC3 were able to upregulate NKp46 and convert to an ILC1-like phenotype and they secrete IFN γ during chronic states of inflammation (78–80). The development of these NKp46⁺ ex-ILC3s requires the downregulation of ROR γ t and induction of TBET. The conversion of an ILC3 population to an ILC1-like cell also requires Notch signaling (80). These ex-ILC3 ILC1-like cells do not develop in ROR γ t-deficient mice further demonstrating their ILC3 origin. Their dependence on ROR γ t suggests that these cells are not ILC1s but should be considered ILC3s since the majority of ILC1s are ROR γ t fate-map negative (10). Interestingly, Th17 cells have been shown to upregulate TBET when treated with IL12 or IL23 and convert to cells that produce both IL17 and IFN γ . These Th1-like Th17 cells are pathogenic in the context of Experimental Autoimmune Encephalitis and have been implicated in other autoimmune disorders but can also be protective in some models of cancer. Ex-ILC3's appear to be different from Th1-like Th17 cells in that they do not retain the expression of ROR γ t. TBET can repress expression of *Rorc*, and in Th1-like Th17 cells this ability may be prevented by high expression of RUNX family transcription factors (81). Therefore, the transcriptional program generating ex-ILC3 may differ in some subtle ways from Th1-like Th17 cells that allows for repression of *Rorc* by TBET; however, these mechanisms remain to be investigated in ILC. IL7 and the microbiota can stabilize ROR γ t expression in NCR⁺ ILC3 preventing conversion to the ILC1-like cell that secrete IFN γ , while IL15 and IL12 contribute to the down regulation of ROR γ t in NCR⁺ ILC3s (82). Thus, environmental signals can also affect ILC development and function.

A common ILC progenitor

NK cells and ILCs all develop from the common lymphoid progenitor (CLP). Recently, Constantinides *et al.* (9) identified a PLZF expressing progenitor in the bone marrow (BM) and fetal liver that gives rise to ILC1s, ILC2s, and ILC3, but not LT α i cells or NK cells, T, or

B cells. Interestingly, although the PLZF⁺ ILC progenitors (ILCPs) did not give rise to LT_i and conventional NK cells, they did express TOX, which is required for both LT_i and NK cell development. PLZF is expressed in ILCPs and plays a role in the development of ILC1s, ILC2s, and ILC3s, since these cells fail to develop from PLZF-deficient progenitors in mixed BM chimeras. However, PLZF is not expressed in mature ILCs indicating that transient expression in ILC progenitors is required to support their development. A recent report from the Diefenbach group (10) identified an Id2⁺α4β7⁺CD127⁺CD25⁻CD244⁺CD27⁺Flt3⁻ bone marrow cell population that give rise to LT_i cells, in addition to ILC1s, ILC2s, ILC3s. This progenitor was named the common progenitor for all helper-like ILCs progenitors (ChILP) and maybe composed of PLZF^{pos} and PLZF^{neg} compartments. Since the ChILP can give rise to LT_i cells as well as ILC1s, ILC2s, and ILC3s it was suggested that the PLZF⁺ subset is downstream of the PLZF⁻ subset. Importantly, the ChILP population is not reduced in IL7Rα^{-/-} mice consistent with data showing the ILC1 cell development is independent of IL7. Importantly, ChILPs did not give rise to conventional NK cells indicating that the NK cell lineage has a distinct developmental pathway that diverges prior to the ChILP stage. This finding is consistent with the observation that some mature NK cells develop in *Id2*-deficient mice (37).

The existence of a ChILP that lacks NK potential is also consistent with recent studies that revealed a role for GATA3 in the development of all CD127⁺ ILCs including LT_i cells, whereas GATA3 is not essential for the development of conventional NK cells (66). These observations suggest that during differentiation of CLPs *Gata3* expression is up regulated coincident with the loss of NK potential. This GATA3-dependent progenitor then retains only LT_i and ILC potential. It was recently shown that one critical function for the B cell transcription factor EBF1 is repression of *Gata3* in CLPs (83), thus preventing either T lymphocyte or ILC development from these cells. EBF1 has also been implicated in the repression of *Id2* and *Nfil3*, indicating that it may play a role in limiting all innate lymphoid potential in the progeny of CLPs destined for the B lymphocyte lineage (84, 85). In this proposed developmental scheme, the innate lymphoid fates would be the “default” pathway from CLPs with upregulation of GATA3 and PLZF promoting the ILC fates and yet to be determined factors controlling specification to the NK cell lineage. A molecular understanding of how NK cell development progresses from CLPs independent of ILCs remains to be determined.

Innate-like T lymphocytes

Multiple evolutionarily conserved innate-like T-cell populations are present in mice and humans that differ in their antigen receptor expression and their functional properties (86). In mice, type I, or invariant NKT cells (also known as iNKT), are the most abundant innate T-cell population, and these were first identified by their rapid expression of cytokines and their expression of multiple NK cell associated surface receptors (87). iNKT cells express an invariant Vα14Jα18 TCRα chain that can pair with a limited number of TCRβ chains (Vβ2, Vβ7 and Vβ8.1–8.2). In humans, iNKT cells express the homologous Vα24Jα18 TCRα, preferentially paired with Vβ11 (human homologue of mouse Vβ8 chain) (88–90). These cells can recognize glycolipid antigens presented by the non-classical, non-polymorphic MHC I family molecule CD1D (91, 92). α-galactosylceramide (αGalCer), isolated from the

sea sponge *Agelas mauritanus*, was the first identified glycosphingolipid that can interact with and strongly activate iNKT cells (93). In addition, there is another population of CD1D-dependent NKT cells with more diverse TCR usage, including non-canonical V α 3.2J α 9/V β 8 and V α 8/V β 8 rearrangements (94, 95). These cells are named Type II or diverse NKTs (dNKT) and most of them preferentially recognize sulfatide, not α GalCer (96, 97). Sulfatide versus glycolipid recognition is a critical distinguishing feature between these NKT subsets, and CD1D tetramers loaded with α GalCer or α GalCer analogs (i.e. PBS57) can be used to specifically detect iNKT cells. Despite their similarities, Type I and Type II NKT cells have distinct functions and in some cases they suppress each other during an immune response (98). A third population of CD1D-restricted NKT cells has been identified recently that expresses the V α 10 gene segment recombined with the J α 50 gene segment, in germline configuration, associated with V β 8 that has a diverse CDR3 β region (99). Although V α 10J α 50⁺ iNKT cells can recognize and be activated by α GalCer, they interact with α GlucosylCeramide (α GlcCer) lipid antigen with higher affinity, thus distinguishing them from V α 14J α 18⁺ iNKT cells. Furthermore, in humans, a population of CD1D-restricted V δ 1⁺ $\gamma\delta$ T cells that can interact with sulfatide or the prototypical glycolipid antigen α GalCer has been recently identified (100, 101).

Additional innate-like T-cell subsets exist that are not dependent on CD1D presentation. These include the MR-1-restricted mucosal associated invariant T cells (MAIT), which express an invariant V α 19J α 33 TCR α chain (V α 7.2J α 33 in humans) specifically recognizing vitamin B metabolites (102), V γ 1.1V δ 6.3⁺ $\gamma\delta$ T cells, whose ligand is still unknown (103, 104, 105), and innate-like CD8 T cells, whose function is unclear (106, 107). Among all innate-like T-cell subsets, type I V α 14J α 18 iNKT cells have been extensively studied.

iNKT cell function and tissue localization

After microbial infection, iNKT cells can be activated by direct presentation of endogenous and/or microbial lipids from dendritic cells (DCs), through CD1D-dependent TCR interactions (6). iNKT cells can enhance DC stimulation, through cytokine production, thus indirectly modulating adaptive T-cell responses (108, 109). Alternatively, iNKT cell activation can be accomplished by stimulatory cytokines, such as IL12, released by DCs following Toll-like receptor (TLR) signaling. Although TLR-stimulated DCs can synthesize and present self-lipids to iNKT cells, the release of pro-inflammatory cytokines in the microenvironment may be sufficient to promote an iNKT cell response, even in the absence of TCR-CD1D interactions (110). Activation of iNKT cells, therefore, involves a combination of innate and adaptive pathways, allowing them to respond to danger signals, even in the absence of foreign antigen recognition. Those distinct activation modes, in combination with the type of lipid antigen and the type of antigen-presenting cell can severely influence the iNKT cytokine production and the ensuing effector function. Their ability to produce a diverse array of cytokines rapidly after infection, allows iNKT cells to modulate the response of other immune cells, such as NK, B, and T cells (111). Due to their broad influence during an immune response, iNKT cell function has been positively or negatively involved into a wide range of diseases, including microbial and viral infection,

cancer, inflammation, and autoimmunity. The effect of iNKT cells has been extensively reviewed elsewhere and is out of the scope of this review article (112, 113).

Unlike conventional T cells, iNKT cells are largely absent from the blood circulation and the lymph nodes and are long-term residents mainly in non-lymphoid and mucosal tissues. Mature iNKT cells are found in the spleen at a frequency of approximately 1% of total lymphocytes, where they are located primarily near blood vessels and within the marginal zone, but not in the white pulp (114, 115). Splenic iNKT cells can be activated by MZ-resident macrophages and DCs and subsequently produce cytokines within hours after the initial antigen encounter. In the mouse liver, iNKT cells are abundant, consisting almost 40% of the total lymphocyte population. Hepatic iNKT cells are retained in the liver sinusoids via LFA-1 and ICAM-1 interactions with the epithelial cells (116, 117), where they can sense blood-borne pathogenic antigens such as *Borrelia* and *Ehrlichia* inoculated through tick bites (118, 119). Similarly, iNKT lung residents are located within the intravascular network in close proximity with the peribronchiolar and interalveolar capillaries and are extravasated upon airborne exposure to lipid antigens (120). iNKTs are also found in the bone marrow, the skin and the gastrointestinal tract. Neonatal exposure to commensal microbiota can regulate the intestinal and lung iNKT population throughout life and protect against NKT-mediated gut inflammation and allergic asthma (121). This is of interest because in humans, iNKT cells are enriched in the omentum, where they consist about 10% of lymphocytes and are significantly reduced in patients with colorectal carcinoma or obesity (122). Collectively, these findings indicate that iNKT cells are strategically located to quickly screen and respond to blood-borne or air-borne lipid antigens that can act as microbial danger signals and interact with the commensal microflora at mucosal surfaces. This is consistent with their function during the innate phase of an immune response and possible interactions with other ILC populations residing in the same tissues may be critical for orchestrating a proper adaptive response.

Development of iNKT cells

iNKT cell selection

The generation of CD1D tetramers loaded with α GalCer or α GalCer analogs, which can specifically interact with iNKT cells, greatly facilitated the study of their developmental pathway (123, 124). Indeed, unlike ILCs, iNKT cell development can be tracked from the moment they initiate expression of the V α 14J α 18 containing receptor, resulting in a more detailed understanding of the mechanisms leading to selection and lineage diversification in this innate-like T-lymphocyte pathway. Similar to classical MHC-restricted T cells, iNKT cell development is thymus-dependent, as these cells are absent from athymic mice and young thymectomized mice (125–127). iNKT cell development branches off from conventional T cell development at the stage of CD4⁺CD8⁺ [double positive (DP)] progenitor cells (128). Expression of the canonical V α 14J α 18 rearrangement in DP cells occurs during stochastic *Tcra* locus recombination events and it instructs the iNKT cell fate, as shown in *Val4Ja18* TCR transgenic models (129–131). In the *Tcra* genomic locus, V and J gene segments recombine sequentially from the most proximal locations towards the more distal. The proximal gene segments recombine early during the life of DP thymocytes,

whereas recombination of distal elements requires longer DP survival. This is an essential checkpoint in iNKT cell development, because the V α 14 and J α 18 gene segments are located distally in the *Tcra* locus; therefore, only long-lived DP cells will have the opportunity to express the iNKT TCR, a finding that can explain the rarity (around 1/100,000 cells) of the iNKT DP precursors. Indeed, genetic mutation of genes encoding proteins that regulate DP lifespan lead to severely reduced iNKT cell numbers (27, 128, 132).

Cells expressing a productive iNKT TCR on their surface undergo a distinctive positive selection process that is tied with the innate phenotype of these cells in the thymus. Expression of CD1D in cortical DP thymocytes (driven by the proximal Lck promoter in *Cd1d*-deficient background) is sufficient for iNKT cell development, whereas bone marrow chimeras that lack CD1D expression specifically on DP cells are unable to generate iNKT cells (133, 134). These results show the specific requirement of CD1D expression in DP thymocytes for the positive selection of iNKT cells. Although it is widely thought that CD1D can present self-lipid antigens to iNKT cells during selection, the identity of the selecting ligand is still unclear. One recent study described that peroxisome-derived ether lipid antigens [i.e. plasmalogen lysophosphatidylethanolamine (pLPE)] are produced in the thymus and can stimulate thymic iNKT cells. However, deletion of *Gnpat*, the enzyme responsible for synthesizing pLPE in peroxisomes, lead to only a modest decrease in more mature iNKT cells, whereas immature iNKT cell numbers remain unaffected or were even elevated, indicating that there are additional pathways that can mediate iNKT cell selection (135).

Regardless of the selecting antigen, TCR-derived signals synergize with the signaling lymphocyte activation molecule (SLAM) signaling pathway to promote iNKT cell selection. The SLAM family of receptors consists of 6 members, all of which signal through interactions with the adaptor protein SLAM-associated protein (SAP) and are expressed in DP thymocytes (136). Although genetic deficiency of individual SLAM receptors resulted in a modest, if any, decrease in iNKT cell number, elegant bone marrow chimeras showed that combined elimination of LY108 and CD150 (SLAM) lead to severe iNKT cell reduction (137). Consistent with a combinatorial role for SLAM receptors in iNKT cell selection, mice lacking the SLAM associated adaptor protein SAP are largely devoid of iNKT cells, with the few remaining cells having an immediate post-selection phenotype (138, 139). Notably, both NOD mice that have decreased CD150 and LY108 expression due to genetic alterations in the respective gene loci and humans with X-linked lymphoproliferative disease (XLP), which results from loss-of-function mutations in the *Sap* gene, lack iNKT cells (140). Although the mechanism is not clear, it has been proposed that SAP blocks an inhibitory signal from the LY108 receptor (possibly mediated through the phosphatase SHP-1), because double SAP- and LY108-deficient mice show increased iNKT cell numbers compared to SAP-deficiency alone (141).

PLZF: a specifying transcription factor for the iNKT cell lineage

A coronal feature of iNKT cells and innate-like T cells in general is the expression of the signature transcription factor promyelocytic leukemia zinc finger (PLZF) (142, 143). As

discussed above, PLZF was recently identified in a precursor to multiple ILC programs and it is expressed in some myeloid cells. PLZF belongs to the broad complex tramtrack bric-a-brac zinc finger (BTB-ZF) transcription factor family, members of which can regulate several immunological developmental pathways, including germinal center formation (BCL6) (144) and CD4 lineage commitment (ThPOK) (145). In the thymus, PLZF is expressed in iNKT and V γ 1.1V δ 6.3 $\gamma\delta$ T cells (103–105, 142, 143). Deletion of, or a loss-of-function mutation in, the *Zbtb16* gene (encoding PLZF) leads to a dramatic decrease in iNKT cell numbers in the thymus, with a concomitant loss of their innate effector phenotype, their ability to rapidly produce cytokines, and their distinct tissue localization. Importantly, conventional polyclonal T cells acquire iNKT-like characteristics when PLZF is ectopically expressed under a CD4- or a pLck-driven promoter, including the ability to co-produce both IL4 and IFN γ and homing to the liver and the lung, even in the absence of TCR or SLAM-SAP signaling or cell division (117, 142, 143, 146, 147). Therefore, PLZF is not only required for iNKT cell development, but is also sufficient to confer innate-like characteristics to conventional T cells. PLZF is, thus, considered a master transcription factor that can specify the iNKT cell fate, although its molecular targets have not been identified yet.

Consistent with the notion that iNKT cells are agonist-selected, immediately post-selection iNKT cells show elevated levels of GFP expression (compared to Class I or Class II MHC-selected T cells) in a TCR-signaling reporter mouse model, suggesting that they exhibit stronger TCR signaling than conventional CD4 T cells (148). PLZF induction can be detected immediately after positive selection of iNKT cells raising the hypothesis that strong TCR signals are responsible for the selective expression of PLZF in iNKT cells. Consistent with that hypothesis, anti-TCR treatment is sufficient to induce high levels of PLZF in T cells, both *in vitro* and *in vivo* (103, 149). Moreover, iNKT cells show elevated and sustained expression, compared to conventional T cells, of the immediate early transcription factor EGR2, which is induced through a TCR/Calcineurin/NFAT-dependent pathway. EGR2 can directly bind to the *Zbtb16* gene promoter activity, thus providing a direct molecular link between strong TCR signals and iNKT cell-specific PLZF expression (149–151). While TCR stimulation alone is sufficient to promote PLZF induction, co-stimulation of the SLAM family receptor LY108 contributes to its maximal expression, by amplifying TCR signaling and EGR2 expression (152). Therefore, the iNKT cell-specific expression of PLZF is achieved through the synergistic activation of the TCR and SLAM receptor signaling pathways. Indeed, forced expression of MHC Class II on cortical DP thymocytes results in a polyclonal SAP-dependent CD4 T population that has high PLZF expression and iNKT-like effector properties (153–155). Therefore, PLZF induction in iNKT cells and the subsequent acquisition of their activated/effector characteristics are the consequence of the distinct positive selection process that these cells undergo.

iNKT cell thymic differentiation

Once iNKT cells are selected, they undergo a step-wise differentiation process in the thymus that results in mature iNKT cells with multiple effector fates that parallel those in ILCs and the CD4 T cell lineage (11, 156) (Fig. 1). However, unlike other ILCs, the earliest stages of iNKT cell development can be clearly identified because of their unique expression of the

V α 14J α 18 containing TCR. The most immature and low abundance iNKT cell population expresses high levels of CD24 and retains expression of both CD4 and CD8. These cells are termed stage 0 (ST0) and represent immediate post-selection iNKT cells, showing high levels of CD69 (157). As cell differentiation progresses, iNKT cells down regulate CD24 (indicative of cell maturation) and CD8 and show low detectable levels of the memory T cell marker CD44 [stage 1 (ST1)]. Although at this point iNKT cells are still characterized by a naïve phenotype, they are able to rapidly produce high levels of cytokines, particularly the Th2 cytokine IL4. As CD44 expression increases and these cells acquire an effector phenotype (stage 2, ST2), they gain the ability to produce Th1- and Th17-associated cytokines, including interferon γ (IFN γ) and IL17, respectively (156, 158, 159). ST2 iNKT cells can terminally differentiate in the thymus and form a long-lasting thymic resident population, with Th1-like features (stage 3, ST3), also known as NKT1 cell subset (Figure 1). ST3 iNKT cells are characterized by upregulation of NK-related molecules, including the beta chain of the receptor for IL15/IL2 (CD122) and NK1.1 in C57BL/6 mice, and in this respect more closely resemble NK cells and ILC1 than CD4⁺ Th1 cells. However, iNKT cells with a CD44^{hi} surface phenotype can either exit the thymus and further differentiate in the periphery, or remain in the thymus as Th2-like, (NKT2) or Th17-like (NKT17) terminally differentiated cell subsets (see below). Whereas ST0/1 cells are uniformly CD4⁺, some developing iNKT cells downregulate CD4 expression; therefore mouse iNKT cells can be either CD4⁺ or CD4⁻, although the mechanisms involved in this fate decision and the functions of these subsets are not clear. This particular developmental scheme is also accompanied by a massive intrathymic expansion that is initiated at ST0 and is largely terminated by ST2, as ST3 cells show no appreciable BrdU incorporation. The IL7 receptor signaling pathway and the oncogenic transcription factor *c-myc* are critical for this expansion phase. iNKT cells from mice lacking *Il7ra* or *c-myc* are reduced in numbers and show impaired proliferation at the immature stages, a phenotype that cannot be rescued by provision of survival signals (160, 161). However, how these factors are regulated in iNKT cells has not been determined. All these features are consistent with a role for iNKT cells during the initial innate phase of an immune response that requires high cell numbers and ample cytokine production to rapidly exert their effector functions. This is in sharp contrast to the developmental pathway of conventional T cells, which generates naïve lymphocytes that require antigen and cytokine stimulation prior to clonal expansion and effector function.

iNKT cell subsets

The segregation of iNKT cells into stages based on expression of cell surface receptors has been a useful strategy for investigating their development and function. However, recent studies revealed that these populations do not exclusively define cells with a precursor progeny relationship, but rather, each stage includes polarized effector subpopulations (11, 162). At least three thymic iNKT cell effector subsets have been identified (Figure 1). Similar to conventional CD4 T cells and ILCs, these subsets are defined based on the expression of signature transcription factors TBET, GATA3 and ROR γ t. Consistent with their Th1-like properties, NKT1 cells that are largely found among ST3 cells express and are dependent on the Th1 signature transcription factor TBET. Th2-like iNKT2 cells that have a ST1/ST2 phenotype express high levels of GATA3, and the Th17-like iNKT17 cells are ROR γ t⁺ cells with a ST2 phenotype. Therefore, ST2 cells are a heterogeneous population

that consists of developmental intermediates of ST3 NKT1 cells and terminally differentiated NKT2 and NKT17 cells. These subsets also display differential expression of CD4, with NKT1s being CD4⁺ and CD4⁻, NKT2 being predominantly CD4⁺, and NKT17 are mostly CD4⁻. Importantly, splenic iNKT cells can express the T-cell follicular helper (Tfh)-associated transcription factor BCL6 upon activation and contribute to B-cell activation and germinal center initiation (163–165), whereas upon TGFβ treatment *in vitro*, iNKT cells can upregulate FOXP3 and suppress CD4⁺ T-cell proliferation, thus resembling regulatory CD4⁺ T cells (166).

Notably, the polarization of the iNKT effector subsets is not as absolute as that of the conventional CD4⁺ T cells or the ILCs, since iNKT cells have the unique ability to produce both Th1 and Th2-associated cytokines at the single cell level. This property is implemented by PLZF, because ectopic expression of PLZF can induce the production of an unbiased pool of cytokines in conventional T cells (143). Intriguingly, the different thymic iNKT cell subsets are characterized by varying levels of PLZF, although it is currently unknown how PLZF expression is regulated in these cells. NKT1 cells have the lowest expression of PLZF (PLZF^{low}TBET⁺), whereas NKT2s are high for PLZF (PLZF^{hi}GATA3^{hi}) and NKT17s are intermediate (PLZF^{int}RORγt⁺) (11). Therefore, although all iNKT cells are under the influence of a PLZF-dependent transcriptional program, polarization of their effector properties is skewed by the simultaneous expression of classical Th signature transcription factors.

In C57BL/6 mice, iNKT cell subsets can also be identified based on the surface expression of IL17RB, a member of the IL25 receptor family. Early studies showed that CD4⁺IL17RB⁺ iNKT cells produce large amounts of Th2 cytokines, including IL4 and IL13 in response to IL25 and can promote allergic reactions and airway hyperreactivity in mice, whereas CD4⁻IL17RB⁺ iNKT cells produce IL17 and IL22 (162, 167). In contrast, IL17RB⁻ cells are predominantly IFNγ producers and IL17RB expression is anti-correlated with CD122 and NK1.1, both features of the NKT1 cell subset. Consistent with their functional potential, CD4⁺IL17RB⁺ iNKT cells express slightly higher levels of GATA3, CD4⁻IL17RB⁺ iNKT cells express RORγt, and IL17RB⁻ cells express TBET (162). Whereas NKT1 cells are dependent on IL15 receptor signaling and are lost in *Il2rb*^{-/-} mice (encoding for CD122), NKT2 and NKT17 cells are largely dependent on IL17RB, suggesting that distinct signaling pathways are required for the development of these different effector fates. Importantly, *in vitro* differentiation experiments showed that bulk ST1 IL17RB⁺ cells could only give rise to ST2 IL17RB⁺ cells, but not to IL17RB⁻ cells, whereas ST2 IL17RB⁺ remained ST2 IL17RB⁺. In contrast, ST1 and ST2 IL17RB⁻ cells converted into ST3 IL17RB⁻, but not into IL17RB⁺ iNKT cells (162). Similar experiments using reporter mice that express RORγt-driven GFP, revealed that RORγt⁺ cells represent a stable IL17-producing iNKT subset (159). These results suggest that iNKT effector fates are stable and that commitment occurs at a very early stage during iNKT cell differentiation with distinct precursor subsets being distinguished based on CD4 and IL17RB expression. However, it is unclear how IL17RB expression is induced after iNKT cell selection. The conclusion that iNKT cell effector fates are stable, and distinguished early, was further supported by intrathymic transfer experiments with KN2 (which reports IL4 production) and TBET reporter mice,

which showed that NKT2 cells could be separated from NKT1 precursors on the basis of IL4 reporter expression (11). Although these studies shed some light on the branch point between NKT1 and NKT2 cells, it is currently under investigation how the NKT2 and NKT17 effector fates resolve in the thymus.

While the NKT1 cell population is predominant in mice of the C57BL/6 strain, other mouse strains, including Balb/c and DBA2 mice, show increased numbers of NKT2 and/or NKT17 cells (11, 106, 168). Furthermore, the relative frequency of NKT1s and NKT2s is inversely correlated in all the mouse strains tested, whereas NKT17s are variable. Not only were more NKT2 cells in Balb/c and DBA2 mice, but they are also capable of producing higher concentrations of IL4 than the C57BL/6 mice, at steady state. Strikingly, strains with high frequency of NKT2 cells contain a considerable amount of innate-like thymic CD8 cells, a phenotype that is dependent on IL4 produced by PLZF-expressing innate-like T cells (106, 107), increased IgE production from B cells and a thymic dendritic cell (DC) population with a Th2-prone chemokine receptor profile (11). A similar phenotype has been described in several mutant mouse strains in the C57BL/6 genetic background, including loss-of-function mutations in the TCR signaling molecules ITK (106), and SLP76 (46), the TCR-induced proteins ID3 (107), KLF2, and the histone acetyl-transferase CBP (106) as well as in mice with stabilized β -catenin (169). Although it is not clear whether all these molecules operate in the same pathway, it is notable that NK1.1 expression and thus NKT1 development depends on sustained CD1D-TCR interactions (170). Indeed, EGR2 can bind and control the expression of *Il2r β* , which is required for the emergence of NKT1 cells, and loss of this population is observed in EGR2-deficient mice (149).

Transcriptional regulation of iNKT cell subsets

Studies using mouse knockout models of the lineage-specifying transcription factors have revealed some aspects of the mechanisms controlling iNKT subset diversification. Early studies showed that *Tbx21*^{-/-} (TBET) iNKT cells lack all NK1.1⁺ NKT1 cells, a phenotype accompanied by a loss of CD122 expression (171, 172). Although, re-expression of TBET lead to CD122 expression, thus showing that TBET may regulate NKT1 development through CD122 expression, it is not clear whether CD122 expression alone can fully rescue the TBET-deficient phenotype. Interestingly, TBET-deficient mice show an expanded population of NKT2 and NKT17 cells and display the IL4-mediated innate-like CD8 phenotype (11). This phenotype is due to a cell-intrinsic requirement for TBET, and not due to competition for space between NKT1 and NKT2 populations, as shown convincingly by a series of unequally competitive bone marrow chimeras. Conversely, the NKT2 population is dependent on the Th2-master transcription factor GATA3, whereas the development of the NKT1 fate is GATA3-independent (173). However, GATA3-deficient thymic NKT1 cells were exclusively CD4⁻, suggesting that CD4⁺ NKT1 cells require GATA3, and NKT1 cells were reduced and dysfunctional in the periphery, indicating that GATA3 can influence the function of all NKT subsets.

Unfortunately, the impact of ROR γ t on iNKT sublineage differentiation has been more challenging to address, due to its widespread requirement on DP thymocyte survival (174). The short lifespan of ROR γ t-deficient DP cells leads to decreased distal rearrangements;

consequently, all iNKT cells are absent in these mice (128). The recent generation of PLZF-driven Cre mouse models can be used to bypass the ROR γ t requirement for DP survival and thus study the effect of this transcription factor in post-selection iNKT cell differentiation. Recent results showed that ThPOK, a BTB-ZF transcription factor crucial for conventional CD4 lineage commitment, which is expressed in all iNKT cells, can suppress the NKT17 fate, by directly repressing *Rorc* gene transcription (175, 176). Interestingly, GATA3-deficient iNKT cells show diminished expression of ThPOK, raising the possibility that GATA3 can indirectly regulate the NKT17 sublineage, although the phenotype of NKT17 cells in GATA3-deficient iNKT cells was not tested (177). Regardless, there is a functional interplay among TBET, GATA3 and potentially ROR γ t and ThPOK that contributes to the determination of iNKT cell effector fates.

ID and E proteins in iNKT cell specification

During thymic development, E proteins are essential to enforce positive selection of classical MHC-restricted T cells, by controlling *Tcra* locus accessibility and rearrangements, and survival of pre-selected DP thymocytes (27, 178). Upon formation of a productive TCR and positive selection signals, E protein activity is reduced, through induction of ID3, leading to cessation of gene rearrangement and progression toward the conventional CD4⁺ and CD8⁺ T-lymphocyte programs (23, 179). Indeed, compound deletion of E2A and HEB at the DP stage can bypass the requirement for TCR-dependent positive selection and leads to the generation of CD8⁺ T cells without TCR expression (178). Consistent with that observation, *Id3*-deficient mice exhibit impaired positive selection in TCR transgenic models, a phenotype that is rescued by simultaneous elimination of E2A (28). These findings led some investigators to propose that the ID3/E protein pathway acts as an early sensor of TCR signaling to control CD4⁺ and CD8⁺ T-cell development and thus adaptive immune responses (180).

Recent studies from our laboratory and others showed additional requirements for ID and E proteins in the development of innate-like T-cell lineages. By using conditional knockout mice to delete HEB or E2A in DP cells, 'D'Cruz *et al.* (27) showed that iNKT cells fail to develop specifically in the absence of HEB but not E2A. Loss of HEB severely affected the survival of DP thymocytes, through downregulation of *Rorc* and *Bclxl* expression. In accordance with the decreased DP lifespan, analysis of *Tcra* gene rearrangements revealed that HEB-deficient cells lacked rearrangements of distal V α -J α segments, including the canonical V α 14J α 18 iNKT TCR recombination. Interestingly, expression of a pre-rearranged V α 14J α 18 TCR chain, that can bypass the requirement for rearrangements, partially rescued iNKT cell development in the absence of HEB. Subsequent studies uncovered that HEB can bind to the promoter region and potentially regulate expression of the iNKT specification factor PLZF, immediately after positive selection, suggesting multiple layers of regulation of this developmental process. Consequently, iNKT cell maturation was also impaired in the absence of HEB (181).

Consistent with these findings, we discovered that ID3 restricts the number of iNKT cells (182). Analysis of mice with a germ line deletion of *Id3* revealed an increase in the number of iNKT cells in the thymus. This phenotype is evident at the earliest stages of iNKT cell

development, with an increased number of post-selection PLZF⁺ iNKT cells with a DP surface phenotype. Given the antagonistic relationship between ID3 and E proteins, we hypothesized that increased E protein activity in the absence of ID3 enhanced iNKT cell development. Indeed, we found that *Rorc* and *Rag*, but not *Bclxl* or *Bcl2* gene expression were maintained in a subset of *Id3*^{-/-} post-selected DP cells. Additionally, we observed increased representation of distal rearrangements, including the canonical iNKT TCR rearrangement. These results are consistent with sustained E protein activity in post-selection TCR-signaled DP thymocytes, which would allow for additional secondary rearrangements, thus increasing the probability of generating an iNKT-specific TCR. In support of this hypothesis, provision of the pre-rearranged V α 14J α 18 TCR α chain in DP progenitors resulted in equal numbers of iNKT cells in WT and ID3-deficient mice. Surprisingly, and despite the strong TCR signals emanating from post-selection iNKT cells, ID3 induction in these cells is less robust than in MHC-selected DP cells. Collectively, these results indicate that increased E protein activity is permissive for iNKT cell selection and specification. In contrast, E protein activity is reduced in response to MHC-derived positive selection signals to allow differentiation toward conventional T-cell lineages.

Interestingly, ID3-deficient mice have a massive expansion of V γ 1.1V δ 6.3 $\gamma\delta$ T cells, indicating aberrant development of several PLZF-expressing thymic populations (104). Although the stage at which ID3 acts during V γ 1.1V δ 6.3 $\gamma\delta$ T-cell development is not clear (mostly because their developmental pathway has not been deciphered yet), these cells can clearly expand throughout life and *Id3*^{-/-} mice eventually suffer from hepatosplenic $\gamma\delta$ T-cell lymphomas, suggesting a failure in controlling cellular proliferation (30). It remains to be determined whether ID3 can also affect MAIT cell development, another PLZF⁺ T-cell subset but this seems likely.

Given the high homology between ID3 and ID2 proteins, the similarity in their biochemical properties and binding partners and the essential role of ID2 in the development of non-T innate lymphocytes, we also interrogated the contribution of ID2 during iNKT cell development. Surprisingly, single deletion of ID2 had no effect on thymic iNKT cell development, presumably because elevated ID3 can act as a compensatory mechanism (182, 183). Therefore, we explored the combinatorial functions of ID2 and ID3 proteins by generating mice with simultaneous deletion of both ID2 and ID3. Because these proteins are the major ID family members expressed in developing thymocytes, these mice exhibit uncontrolled E protein activity. Consistent with the notion that E protein activity is not antagonistic to iNKT cell development and results in an extended window for *Tcra* recombination, mice deficient for both ID2 and ID3 exhibited a dramatic increase in the number of thymocytes expressing the iNKT TCR (182, 184). In contrast, conventional T-cell development was severely impaired, as CD4 or CD8 T cells were barely detected. These phenotypes were more severe than those observed in the single ID protein knockouts, suggesting that ID2 and ID3 can function redundantly during positive selection. These data indicate that novel requirements for the E and ID proteins emerge concomitant with the bifurcation of conventional and innate-like T-cell lineages in the thymus.

Despite high iNKT cell numbers, ID2/ID3 double knockout mice fail to induce expression of PLZF after iNKT cell receptor selection and have a naive phenotype similar to the PLZF-

deficient mice, suggesting that excessive E protein activity is detrimental for entry into the iNKT cell lineage (182). Therefore, ID proteins act as gatekeepers of iNKT cell specification through optimal regulation of E protein dose, which in coordination with EGR2, is required to control PLZF expression. Interestingly, EGR2-deficient mice have reduced levels of ID2 and EGR2 can bind to *Id2* gene promoter in human NKT cells (149). Additionally, PLZF can directly promote *Id2* expression in myeloid cells (185). Taken together, these observations suggest that EGR2, ID2 and PLZF may form a positive feedback loop that can initiate and subsequently stabilize the innate-like T-cell fate. Therefore, iNKT cell specification requires the orchestration of a transcription factor network that combines features of both adaptive and innate lymphocyte development.

ID proteins and the regulation of iNKT cell effector fate

Given the widespread effect of the NKT2 cells in the development of neighboring immune cells, and thus potentially during an immune response, deciphering the molecular mechanisms involved in this process is imperative. Although the developmental branch point between the different iNKT cell populations has been narrowed down, the molecular mechanisms regulating iNKT effector fate decisions are largely unknown. We, and other groups, have independently provided evidence that ID proteins are major regulators of the iNKT effector fate decisions (181, 182, 186). We showed that ID3-deficient mice lack almost all NKT1 cells, whereas there is an accumulation of NKT2 cells (182). Consistent with this phenotype, there are less *Id3*^{-/-} iNKT that can produce IFN γ than their wildtype littermates and more IL4 or IL4 and IFN γ dual producers. Furthermore, PLZF and GATA3 expression is retained at high levels in the vast majority of ID3-deficient iNKT cells, in accordance with the increased percentage of IL4⁺ IFN γ -producing cells. Consistent with this phenotype, ID3-deficient iNKT cells lack TBET expression both at the mRNA and protein level, although the few ST3 iNKT cells that develop, display normal levels of TBET, showing that ST3/NKT1 cells are strongly selected for proper TBET expression. Surprisingly, ST3 cells show low, ID3 expression, in contrast to ST1 and ST2 cells that are high ID3 expressors; however, ID3 is intrinsically required for NKT1 development, and the lack of NKT1 cells is not due to aberrant NKT2 cell expansion, because *WT* NKT1 cells develop normally in mixed bone marrow chimeras. These results suggest that ID3 is not important for the survival of NKT1 cells or the maintenance of the NKT1-specific transcriptional program; rather it is essential to control the earlier stages of NKT1 cell development, while it is dispensable or restrictive for NKT2 cell development.

To gain further insight into the stage at which ID3 acts to determine the NKT1 cell fate, we performed global transcriptome analysis in ST1 and ST2 iNKT cells from ID3-deficient and -sufficient mice. Our analysis revealed that the majority of genes that are progressively upregulated from stage 1 to stage 3 (NKT1-associated gene expression program) failed to initiate expression during the stage 1 to 2 transition in *Id3*^{-/-} iNKT cells. This de-regulation is already evident when comparing ST1 cells, where 50% of the NKT1-associated genes show reduced expression in the absence of ID3. These genes include the master NKT1 regulator TBET, the cytokine gene IFN γ and its receptor, NK-associated receptors and components of the IL15, IL12 and IL18 receptors. Importantly, the number of ST1 IL17RB⁻ (NKT1 progenitors) and IL17RB⁺ (possibly containing a mixture of mature and immature

NKT2 cells) were increased in *Id3*^{-/-} mice compared to *WT* littermates, indicating that NKT1 progenitors are present even in the absence of ID3 (authors' unpublished observations). However, *Tbx21* expression is severely reduced in these cells, corroborating our results that ID3 affects the initiation of the NKT1-related transcriptional program. Furthermore, *Id3*^{-/-} mice display a severe reduction in ST2 IL17RB⁻ cells with a concomitant increase in ST2 IL17RB⁺ (the large majority of which are mature NKT2 cells), indicating impaired progression of NKT1 progenitors through differentiation. Importantly, IL17RB expression at the single cell level is comparable between *WT* and *Id3*^{-/-} iNKT cells, suggesting that ID3 does not control NKT2 cell fate through modulation of IL17RB expression. Collectively, these results suggest that ID3 loss is inhibitory for NKT1 cell differentiation, whereas it is permissive for NKT2 cell generation.

While deletion of ID3 severely affects *Tbx21* expression, it is still not clear whether TBET and ID3 operate within the same molecular pathway to control NKT1 cell development. Gene expression analysis of ST1 and ST2 cells showed that ID3 is not affected in the absence of TBET (authors' unpublished results), indicating that ID3 may act either upstream or in parallel pathways with TBET to regulate NKT1 cell commitment. E protein homodimers are thought to act as transcriptional activators, rather than repressors; therefore, it is possible that excess E protein dose can activate a transcriptional repressor that in turn can regulate TBET expression. Alternatively, E proteins can heterodimerize with some Class II bHLH proteins that function as transcriptional repressors (187). Importantly, iNKT cells express the Class II bHLH protein BHLHE40 that can repress transcription and has been shown to interact with E2A. However, direct molecular evidence to distinguish between these possibilities is currently lacking.

Independent studies from other groups, using different genetic models to eliminate ID proteins (181) or titrate E protein dose (186), revealed additional requirements for ID and E proteins during iNKT cell development. Goldrath and colleagues generated knock-in mouse strains that have GFP or YFP inserted in the coding regions of the *Id3* and *Id2* genes, respectively (*Id3*^{G/G} and *Id2*^{Y/Y} mice). This is a powerful genetic approach, because it accomplishes two goals: *Id3* and *Id2* mRNA expression can be followed by flow cytometry at the single cell level and at the same time, homozygous mice act as loss-of function mutants, similarly to germ line knockout mice. Using this strategy, the authors showed that expression of *Id2* and *Id3* overlaps in ST1 and ST2 iNKT cells, whereas ST3 cells express high levels of *Id2*, but lower levels of *Id3*. Importantly, compound deletion of any 3 of 4 *Id* alleles showed a substantial enrichment in ST1 and ST2 cells, relative to single *Id2* and *Id3* knockouts, accompanied by a loss of ST3 cells. However, this phenotype is less severe than the one observed in double *Id2* and *Id3* knockout mice, where iNKT cell development is blocked at ST1. Although the development of the different iNKT subsets was not assessed in these experiments, the results indicate that a gradient of E protein activity controls the developmental progression of iNKT cells in the thymus.

The studies from the Goldrath group further revealed that in the absence of both E2A and HEB, the early proliferation burst of iNKT cells is impaired, as assessed by staining of the nuclear protein Ki67. Conversely, *Id3* reporter expression was correlated with proliferating iNKT cells and *Id3*^{G/G} ST2 iNKT cells show elevated Ki67 levels, in mixed bone marrow

chimeras. Interestingly, our studies reveal no increased proliferation in the absence of ID3 using BrdU incorporation. Since BrdU incorporation marks actively proliferating cells at the S phase of the cell cycle, whereas Ki67 is ubiquitously expressed in actively cycling cells, but not in G0, it is possible that the increased Ki67 staining may indicate a failure to enter G0. Alternatively, these discrepancies may be explained by the abundance of fetal-derived V γ 1.1V δ 6.3 $\gamma\delta$ T cells in *Id3*^{-/-} mice, whose development is not supported by adult bone marrow chimeras. Importantly, NK1.1⁺ V γ 1.1V δ 6.3 $\gamma\delta$ T cells can compete effectively with PLZF^{hi} iNKT cells for the same thymic niche, thus limiting their expansion (188). Nonetheless, it is possible that the ID/E protein pathway can influence the proliferation of iNKT cells, in addition to the lifespan of DP cells and the extent of secondary rearrangements.

Alberola-Ila and colleagues (186) followed a different genetic approach to investigate the effect of uncontrolled E protein activity during iNKT cell development. They generated a knockin mouse model where the bHLH domain from the Class II bHLH protein TAL1 was been fused with the transactivation domain of E47. The TAL1 bHLH domain cannot homodimerize, but has higher affinity for E proteins than ID proteins; therefore the fusion protein, called ET2, can sequester E proteins from the influence of ID proteins, leading to sustained E protein DNA binding. This approach only partially inhibited the ID-E protein interaction because E protein DNA binding in CD3 + CD28 stimulated ET2 DP thymocytes was intermediate between that in pre (low for ID proteins) and post- selection (high for ID protein) DP thymocytes from WT mice. Using CD4Cre mice to conditionally induce ET2 in DP cells, the authors found normal iNKT cell numbers, consistent with partial, or no, ID inhibition. However, ET2 did cause an enhanced development of NKT2 cells and decreased numbers of NKT1 cells, similar to the phenotype of ID3-deficient mice. Interestingly, the number of ET2-expressing iNKT cells is dramatically decreased in the periphery suggesting a problem with thymic emigration. Consistent with that interpretation, neuropilin-1, a receptor associated with thymic emigration (189), was reduced in expression of these iNKT cells. ET2 mice are also characterized by a massive expansion of ROR γ t⁺ iNKT cells that were either IL17RB positive or negative. Although the function or the gene expression profile of these cells was not examined, this finding implies that intermediate E protein doses may promote this cell fate. While ID3-deficient mice do not show this expansion of NKT17 cells it is possible that an increase of E protein activity in ID3 low/negative iNKT cells could influence NKT17 development.

It is surprising that the phenotype of ET2 expressing iNKT cells is not recapitulated in any of the ID-protein deficient models. One possible explanation for this difference is that the TAL1 bHLH domain has subtly different consensus binding sequences than the Class I bHLH proteins and therefore may have redirected E proteins to unique target genes. In addition, it is possible that ET2-E protein heterodimers could antagonize expression of genes that are regulated by E protein dimers in iNKT cells. Additional experiments are needed to identify the critical E protein target genes in iNKT cells and to determine how their expression is regulated in these different models of E protein dose. Regardless of the mechanisms involved these independent studies uncovered, for the first time, essential functions for the ID/E protein pathway in innate-like T cell development.

While ROR γ t and GATA3 are expressed at the DP stage, TBET expression is induced after commitment to the iNKT lineage. By ST1, iNKT cells are committed to become NKT1, NKT2 and NKT17 cells, however, it remains unclear how the lineage associated transcription factors are regulated and how commitment occurs. Although TBET is required to promote the NKT1 and suppress the NKT2 cell fate, it is not known whether it is sufficient for NKT1 commitment or whether additional factors are contributing. A plethora of transcription factors and signaling molecules have been shown to affect iNKT effector fate differentiation. The current lack of understanding of direct molecular interactions, due to the low iNKT cell numbers, precludes the formation of hierarchical networks that can determine iNKT cell fate decisions. As whole genome approaches for a small number of cells or even for single cells become more accessible, the delineation of such networks will be accomplished.

The finding that ID2 cannot fully compensate for ID3 indicates that these two highly homologous proteins may have unique functions during iNKT effector fate determination. Although iNKT progenitors express both ID proteins, the terminally differentiated iNKT cell subsets predominantly express only one ID, indicating these proteins are regulated independently (181). The mechanisms that control ID2 and ID3 throughout NKT cell development remain to be determined. Interestingly, ID3 and ID2 are differentially regulated by cytokine signaling during CD8⁺ T-cell effector differentiation and STAT proteins can modulate *Id* gene transcription (190). It is possible that similar pathways act during iNKT effector differentiation. Understanding how *Id* gene expression is controlled in iNKT cells will provide substantial insight into how iNKT cell development and effector fate choice is controlled.

Concluding remarks

In recent years, there has been a tremendous effort to identify subsets of innate lymphoid cells and characterize their developmental pathways and functional relevance. Similarly, while iNKT cells, an innate-like T-cell population, have been characterized for over 15 years, it is now appreciated that they also acquire multiple effector fates and that these fate decisions are developmentally regulated. Intriguingly, for both ILCs and NKT cells these effector fates are functionally and molecularly similar to the well-established conventional CD4 helper subsets. However, how these distinct cell fates are resolved from common progenitors and whether similar molecular mechanisms are involved in each lineage (i.e. ILC versus NKT) remains to be determined. ILCs and iNKT cells share a developmental pathway that involves high expression of ID2 and PLZF in progenitor cells, prior to effector fate resolution, which is distinct from the conventional adaptive lymphocyte pathway or the pathway giving rise to NK cells. The signals implicated in effector fate decisions are currently unknown, but given the recent characterization of the common progenitors, it is likely that these will be identified in the near future. Furthermore, whole-genome transcription factor binding studies for relevant factors, combined with transcriptome and epigenetic analyses are warranted to decipher the combinatorial networks that control effector fate diversification. This quest will also reveal unique molecular players involved in these choices that can be subsequently used to study the unique functions of each subset and possibly to modify their function.

One major area of focus over the next few years will be the necessity of these innate lymphoid cells for protection of the host from pathogenic insult. Regardless of the outcome of these studies, it is clear that NK cells, ILCs, and iNKT cells are potent effector cells that can produce sufficient cytokine to influence the immune response. Therefore, their aberrant function can lead to disease but harnessing the effector capacities could also be beneficial in the treatment of many diseased states including cancer and autoimmunity.

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Fig. 1. Schematic representation of the developmental relationships between the distinct effector fates of conventional T cells, innate lymphoid cells, and iNKT cells

A similar architecture of precursor progeny relationships characterizes the development of T lymphocytes and innate lymphoid cells and innate-like lymphocytes. (A) Post-positive selection T lymphocytes differentiate into naive CD4⁺ or CD8⁺ T cells. Upon stimulation, the naive CD4⁺ T cells can give rise to TBET⁺ Th1, GATA3⁺ Th2 or RORγt⁺ Th17 T-helper subsets. (B) Innate lymphoid cells can similarly differentiate from CLPs into NK cells (analogous to CD8⁺ T cells) or helper (ILC) lineages (analogous to CD4⁺ T cells). In contrast to naive CD4⁺ T cells, ID2 and/or PLZF are expressed in ILC progenitors and may promote acquisition of an effector state in the absence of antigen exposure. (C) iNKT cell progenitors can differentiate into multiple helper lineages analogous to CD4⁺ T cells. PLZF and ID protein activity promote the acquisition of a primed effector fate. ID proteins play additional roles in iNKT cell effector fate determination.

Fig. 2. Gene regulatory networks underlying iNKT and ILC cell development

(A) Reconstruction of gene regulatory networks that control NKT1, NKT2 and NKT17 cell fates from a common iNKT progenitor. A gradient of E protein activity controls both iNKT cell specification and iNKT effector fate decisions. (B) Gene regulatory networks that control NK and ILC development from CLPs. Depicted are genes that define the respective effector cell types and their known regulators. Solid lines indicate direct transcriptional control shown by ChIP and gene expression analysis. Dashed lines indicate transcriptional control through unknown mechanisms, shown only by gene expression analysis. Thick dotted lines indicate post-translational control. (») indicates control of cell fate.