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Orchestration of Invariant Natural Killer T cell development by E and Id proteins

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Abstract

Natural Killer T (NKT) cells are $\alpha\beta$ T cells that express a semi-invariant T cell receptor (TCR) along with Natural Killer (NK) cell markers, and have an innate cell-like ability to produce a myriad of cytokines very quickly upon antigen exposure and subsequent activation. These cells are diverted from conventional single positive (SP) T cell fate at the double positive (DP) stage where TCR-mediated recognition of a lipid antigen presented on a CD1d molecule promotes their selection into the NKT lineage. Although many key regulatory molecules have been shown to play important roles in the development of NKT cells, the mechanism of lineage specification and acquisition of effector functions in these cells still remain to be fully addressed. In this review we specifically discuss the role of a family of class I Helix Loop Helix proteins known as E proteins, and of their antagonists Id proteins in NKT cell development. Recent works have shown that these proteins play key roles in iNKT development, from the invariant TCR rearrangement to terminal differentiation and maturation. Elucidating these roles provides an opportunity to uncover the transcriptional network that separates NKT cells from the concurrently developed conventional $\alpha\beta$ T cells.

Keywords

iNKT; E proteins; Id proteins; development

I. INTRODUCTION

A distinct population of $\alpha\beta$ T cells possessing NK (Natural Killer) cell markers and the innate-like ability to mount a potent immune response within hours of exposure to antigens, is referred to as NKT (Natural Killer T) cells. A unique feature that distinguishes these cells from most conventional $\alpha\beta$ T cells is the ability to recognize microbial and self-lipids presented on the non-canonical CD1d molecule, which is MHC (Major Histocompatibility Complex) Class-I like in structure. It has been found that NKT cells can be activated directly by antigen recognition, or indirectly by APCs (Antigen presenting cells),^{1, 2} to produce a wide range of cytokines. Further, unlike the diverse TCR (T cell receptor) repertoire represented by conventional $\alpha\beta$ T cells, most of these cells express a semi-invariant TCR. The most well characterized subset of these are the type I NKT cells, or iNKTs (invariant

Natural Killer T cells) that express an invariant V α 14-J α 18 TCR α chain paired with primarily V β 8.2, V β 7 or V β 2 chain in mice, or an invariant V α 24-J α 18 V β 11 TCR in humans.^{3, 4} This semi-invariant TCR allows these cells to recognize α -GalCer (a marine sponge derived α -galactosylceramide) among other closely related lipids, which is also utilized for their tetramer-based identification across mice, humans and non-human primates.⁵ The type II NKT cells have more diverse TCR pairings and recognize other CD1d-presented lipids, but will not be focused upon in this review and the term NKT will be used exclusively for iNKT cells. iNKT cells are known to play a contextual role in diseases, as they are found to be protective in infectious diseases, tumors and certain autoimmune diseases but harmful in asthma and allergy.^{6–8} Their ability to cross-activate dendritic cells (DCs) and other immune effectors through cytokines and chemokines has also garnered a lot of attention to their potential as vaccine adjuvants.⁹

Although iNKT cells constitute only a small fraction of T cells in the thymus and periphery of mice and humans, their invariant TCR and recognized ligands have been evolutionarily highly conserved across species, indicating a critical role in the immune system. It is currently accepted that these cells arise from conventional $\alpha\beta$ T cell progenitors and follow the same developmental program until the DP (CD4⁺CD8⁺ double positive) stage, where the stochastic expression of the semi-invariant V α 14-J α 18 TCR (henceforth referred to as iNKT TCR) allows CD1d-mediated selection, bifurcating them from conventional SP (CD4⁺ or CD8⁺ single positive) fate.^{10–12} This developmental pathway is known to be regulated at different stages by several transcription factors including PLZF (Promyelocytic leukaemia zinc finger protein)¹³ and EGR2 (Early growth response 2),¹⁴ but the exact developmental regulatory programs in iNKT cells are far from fully elucidated.

A family of Class I bHLH (basic Helix Loop Helix) proteins known as E proteins can regulate transcription by binding to E-box (CANNTG) domains, and are known to play key roles in both B and T cell development.¹⁵ E protein family members (E2A, HEB and E2-2) dimerize with each other in order to bind DNA. This DNA binding and regulation can be prevented by the formation of heterodimers with members of another HLH (Helix Loop Helix) family known as Id (Inhibitor of DNA-binding) proteins.¹⁵ Id2 and Id3 are the key Id family members that are known to inhibit E protein activity in lymphocytes.¹⁶ In conventional T cell development it is known that E proteins promote lineage commitment and early development, but their activity must be down regulated upon pre-TCR signaling to allow DN to DP transition, and subsequently must be further repressed by Id proteins upon TCR signaling to allow DP to SP transition.^{17–19} E and Id proteins have been previously demonstrated to play roles in NKT development^{20–22} such as the critical role of HEB in iNKT TCR rearrangement²³ and Id2 in hepatic NKT homeostasis.²⁴ Recently, more substantial evidence has been published to indicate various roles of these proteins in NKT lineage commitment, development and function.^{25–28}

NKT cell development is a very broad subject and has been covered in depth in other reviews.^{6, 29–44} Several comprehensive reviews have also been written on NKT antigen recognition,^{45–48} transcriptional regulation,^{29, 49–52} and function.^{7, 53, 54} This review will be limited to discussing key publications on the role of E proteins in NKT development as well as highlighting unanswered questions in the field.

II. iNKT DEVELOPMENT AND REGULATION BY E PROTEINS

Ever since their discovery, the lineage history of NKT cells has been a topic of debate in the field, with researchers supporting either the “pre-commitment” or the “TCR-instructive” model of NKT development.⁴³ The first model suggests that NKT lineage fate is preprogrammed, i.e. an intrinsic NKT-specific program exists in precursors even prior to CD1d-mediated selection, which separates them from conventional T cells. These pre-committed NKT cells then undergo NKT lineage specification upon signaling through the NKT TCR and CD1d-mediated selection.⁵⁵ In support of this theory, V α 14J α 18 rearrangements have been detected prior to development of conventional $\alpha\beta$ T cells during fetal life indicating a possibly distinct NKT developmental pathway.⁵⁶ Also, NKT “precursor” DN4 cells with V α 14J α 18 transcripts (but no surface TCR expression) have been detected in CD1d-deficient mice, and shown to give rise to NKT cells when cultured with normal thymocytes.⁵⁷ This supports the existence of early precursors with a NKT-directed program, and specifically directed NKT TCR rearrangement prior to selection. Recent data from our lab also hints towards the role of E proteins in promoting NKT TCR rearrangement before the DP stage.²⁵ In addition, it has been proposed that the distinct transcriptional machinery required for development of conventional T and NKT cells supports the idea of distinct precursors.^{40, 58} In line with this, it has been found that certain mutations selectively impair NKT development without any impact on conventional T cell development.^{58, 59} It has also been suggested that innate-like $\alpha\beta$ and $\gamma\delta$ T cells follow a different developmental program from conventional T cells, such that the TCR expression of the opposite isotype is not selected against in these cells.⁶⁰ The complete block in conventional T cell development in TCR α –/– animals with a V α 14 transgene again indicates that the NKT TCR may primarily be expressed only in the NKT precursor cells.⁶¹ Thus, data in support of the pre-commitment theory is isolated but substantial.

The TCR-instructive model postulates that DP cells which have stochastically rearranged a V α 14-J α 18 TCR, undergo CD1d-mediated selection and subsequent SLAM/SAP (Signaling lymphocytic activation molecule/ SLAM associated protein) signaling,⁶² that drives their NKT lineage commitment. In favor of this model, there is strong evidence to suggest that the NKT TCR is generated from the same random rearrangement process as conventional T cells, rather than being uniquely regulated as suggested by the pre-commitment model. For instance, the TCR repertoire in NKT cells reflects the same stochastic patterns as conventional TCR α rearrangement.^{3, 63} Furthermore, it has been found that the NKT-specific V α 14-J α 18 rearrangement is also subject to the same rearrangement bias of pairing with proximal V α and J α segments.⁶⁴ This would imply that only secondary rearrangements can give rise to the distally rearranged V α 14-J α 18 TCR. It has been shown that mice deficient in ROR γ t (RAR-related orphan receptor gamma t), an important survival factor for DP cells which prolongs the window of secondary rearrangements,⁶⁵ lack NKT cells due to impaired V α 14-J α 18 rearrangement.^{10, 11} It was further shown that NKT development could be rescued by expression of a Bcl-xL (B-cell lymphoma – extra large) transgene, which increased DP cell survival and hence promoted distal rearrangements.¹¹ NKT cell development has also been shown to consist of a DP intermediate¹², and to proceed through the DP stage using fate mapping techniques.¹¹ It has been proposed that similar to $\gamma\delta$ T

cells, strong TCR signaling is critical for selection into the NKT lineage,⁶⁶ which has also been demonstrated in a reporter mouse.⁶⁷ A role of strong TCR signaling in PLZF expression, a key transcription factor in NKT development, has also been shown.^{14, 68} These observations have garnered great support in favor of the TCR-instructive model. However, it is important to note that both models heavily stress upon the timing of V α 14-J α 18 rearrangement. While it is clear that CD1d-mediated TCR engagement provides the decisive signal for NKT lineage commitment, it still needs to be determined whether all or just a fraction of DP cells are competent to become NKT cells. Re-evaluating available data in this context may serve as a better approach to uncovering the transcription program that distinguishes iNKT TCR from other TCR signals during NKT commitment.

NKT cells are either CD4SP or DN cells that proceed through 4 broad stages of development (figure 1) - commitment (stage 0, CD24^{hi}), expansion (stage 1, CD24^{lo} CD44^{lo}), differentiation (stage 2, CD44^{hi}) and maturation (CD44⁺ NK1.1⁺).³⁰ In this figure we have also marked several “steps” in their development, which may be regulated by transcription factors, particularly E proteins, and can therefore impair or support NKT development. These steps include NKT TCR rearrangement, CD1d-mediated selection and subsequent transitions between stages. For example, EGR2 is found to be important for PLZF expression,¹⁴ while NF- κ B⁶⁹ (nuclear factor kappa-light-chain-enhancer of activated B cells) and c-Myc⁷⁰ are important for the proliferative burst at stage 1. Although the roles of E or Id proteins in NKT development have so far been limited to that of HEB²³ and Id2,²⁴ several key roles have been revealed in more recent papers which are discussed in subsequent sections. Previously, it was thought that stage 1 iNKTs secrete only IL-4 on stimulation, stage 2 cells produce both IL-4 and IFN- γ and stage 3 cells produce mostly IFN- γ , but it has now been shown that 3 terminally differentiated iNKT subsets (iNKT1, iNKT2, iNKT17) similar to the conventional T helper cell subsets exist within these linearly ordered stages of iNKT development, each with distinct cytokine profiles.^{71–74} The role of E and Id proteins in influencing subset differentiation and in other steps (figure 1) of NKT development are summarized (table 1) and discussed in detail in the following sections.

A. iNKT TCR expression and selection into the NKT lineage

After pre-TCR selection at the DN3 stage, the expression of a hallmark V α 14-J α 18 TCR in DP cells and selection by CD1d-presented antigen on other DP cells, allows commitment to the NKT lineage (**step 2**, figure 1). This process of “selection” may be randomly governed by TCR expression, or may be pre-determined by an NKT precursor, depending on which model of development is being considered. In this section below, we will primarily discuss the role of E proteins in influencing NKT TCR rearrangement, but it is worthy of mentioning that consistently high levels of E proteins can impair the pre-TCR checkpoint and limit selection into the $\alpha\beta$ lineage, which would also take a toll on selection into the NKT lineage.²⁵

It has been demonstrated in the past that Id2 deficiency allows normal NKT development, but impairs NKT survival and localization to the liver.²⁴ However, the first concrete evidence of E proteins playing a role in NKT development was the lack of the E protein HEB leading to a severe reduction in NKT cell number at all stages in the thymus and

periphery.²³ Such a phenotype could be explained by the associated reduction of ROR γ t expression, a direct target of E proteins,^{75, 76} and the downregulation in ROR γ t target Bcl-x_L, an important survival factor for DP cells.⁷⁷ As mentioned before, a similar phenotype is also observed in ROR γ t^{-/-} mice due to reduced secondary TCR rearrangements, particularly V α 14-J α 18.¹¹ Another interesting observation made by this group was that even an NKT TCR transgene could not fully rescue development past stage 1, suggesting that HEB may play other roles in influencing NKT differentiation and maturation. It is important to note that E2A and HEB have different expression levels, and hence most likely play different roles during NKT development.²³

In a recent paper, Barbara Kee's group demonstrated that Id3 deficiency led to increased selection into the NKT pathway partially because it allowed continued expression of E protein targets ROR γ t, RAG1 (Recombination activating gene 1) and RAG2 (Recombination activating gene 2).²⁷ Thus, even after positive selection, a fraction of Id3^{-/-} DP thymocytes continued rearranging their TCR, resulting in a larger fraction of cells with the distal V α 14-J α 18 rearrangement. Also, in our recent publication, we have demonstrated that combined deletion of both Id2 and Id3 at the DN3 stage using Lck (lymphocyte-specific protein tyrosine kinase) Cre led to a dramatic expansion of both CD4⁺ and DN iNKT cells.²⁵ Using high-throughput sequencing of the TCR repertoire of pre-selection CD69⁻ DP cells from these mice, we observed that there was random pairing of J α chains in cells with the common V α 8 rearrangement but a positively biased pairing of the J α 18 chain in cells with V α 14 rearrangements, as compared to WT mice. This suggests that the lack of Id proteins or very high E protein levels can perhaps promote a skewed TCR repertoire in favor of selection into the NKT lineage.²⁵

It is important to mention that Id and E protein dosage can influence lineage choice and differentiation in NKT cells. For instance, the NKT population is quite small in WT mice suggesting that low E protein activity after the pre-TCR checkpoint in WT mice is not sufficient to promote selection into the NKT lineage. However it seems that deletion of Id2 and Id3 can enhance the E protein levels sufficiently to allow them to specifically promote the NKT rearrangement and hence promote the NKT lineage fate choice.²⁵ The mechanism by which E proteins promote this rearrangement is however unclear, and may be through binding to V α 14 or J α 18 promoter or enhancer regions. It is also possible that Id proteins suppress this rearrangement instead of E proteins promoting it. There is also potential for exploring the role of HEB in influencing NKT development beyond cell survival and secondary rearrangements, such as through the dysregulation of metabolic genes and cytokines like IL17R β and IL12R β , which can also be expected to play important roles in this lineage.²³

Thus, E proteins are critical for NKT development in order to allow more secondary rearrangements at DP stage, and Id proteins limit selection by preventing the same. However, it still remains to be determined if E proteins are also capable of biasing the lineage decision by specifically regulating NKT TCR expression, and if so, how.

B. Initiation of the iNKT developmental program

Selection into any lineage is coupled with induction of the appropriate transcriptional program, marking “commitment” into the lineage. This program must regulate the appropriate expression of transcription factors needed for NKT development and maturation, and is usually distinctly recognizable by the expression of a “master regulator”. After an intensive screening in search of a unique NKT-specific transcription factor, PLZF was identified as a key regulator of NKT development.^{13, 78} Later it was discovered that NKT cells shared PLZF expression with other innate-like cells, including V γ 1.1V δ 6.3 $\gamma\delta$ T cells⁶⁸ and MAIT (Mucosal Associated invariant T) cells.¹³ PLZF is found to be upregulated soon after TCR signaling in stage 0 and stage 1 NKTs (**step 3**, figure 1).¹³ PLZF expression is critical for NKT development and function, as demonstrated by the decrease in NKT numbers, disturbed cytokine production and NKT localization in lymph nodes in PLZF-deficient mice.¹³ Further, expression of a PLZF transgene leads to production of IL-4, IFN- γ , as well as IL-17 by NKT cells suggesting that it promotes a broad NKT effector program in these cells prior to any subset differentiation.^{13, 28, 71, 79}

A recent study has shown that strong TCR signaling, as is observed in case of NKT selection, immediately causes EGR2 upregulation followed by PLZF expression.¹⁴ The presence of an EGR2 binding site in the PLZF promoter region and the failure to upregulate PLZF in EGR1/2 double deficient mice indicates that TCR signaling mediated EGR2 upregulation leads to PLZF expression in stage 0 NKT cells. On the other hand, another group’s work has demonstrated that E proteins bind to two E-box sites in the PLZF promoter region to directly regulate PLZF expression.²⁶ Sustained E protein expression has also been shown to cause increase in PLZF expression.²⁸ In contrast however, Id2/Id3 double deficient mice have also been found to have a 2 fold reduction in PLZF expression, as well as a naïve NKT phenotype similar to PLZF^{-/-} mice.²⁷ Further, the authors found an increase in EGR2 levels in these NKT cells, implying that Id2 and Id3 are important for PLZF expression in an EGR2-independent manner.

There are several possibilities that can explain these contrasting observations. It is possible that Egr2 positively regulates either E or Id proteins. The fact that TCR signaling promotes Egr2, which lies upstream of Id3 in $\gamma\delta$ T cells,⁸⁰ suggests that Egr2 may downregulate E proteins in these cells. E proteins and Egr2 may also coordinate to promote PLZF, which would explain why loss of either E proteins or Egr2 leads to a similar phenotype. Furthermore, Egr2 deficiency has also been demonstrated to reduce Id2 expression in NKT cells.¹⁴ This could imply that Egr2, Id and E proteins can also regulate PLZF expression at different stages during NKT development. For instance, initially Egr2 and E proteins can promote PLZF together, until Egr2-mediated Id2 expression suppresses E protein to prevent further PLZF upregulation. Alternatively, Id2 and Id3 may positively regulate PLZF expression in the early stages of NKT development. This would be supported by the observation that ET-2 mice with sustained E protein levels throughout NKT development, showed low PLZF in stage 1 but high PLZF in stages 2 and 3.²⁸

While the exact mechanism of PLZF regulation in NKT development is unclear, it is certain that E and Id proteins are critical for appropriate expression of PLZF, and hence, are

important for NKT-specific program initiation. It is important to note that PLZF is also considered important for the development of innate-like V γ 1.1V δ 6.3 $\gamma\delta$ T cells,⁶⁸ and so E and Id proteins can promote both iNKTs and these $\gamma\delta$ T cells. Indeed, our data has shown that reducing E protein levels to 50% lower (Id2^{f/f} Id3^{f/f} E2A^{+f} HEB^{+f} LckCre⁺) than Id2^{f/f} Id3^{f/f} LckCre⁺ mice, led to a drastic change in phenotype from predominantly iNKT cells to mostly V γ 1.1V δ 6.3 $\gamma\delta$ T cells, suggesting that E protein levels can influence lineage choices between these two closely linked cell types.²⁵ The downstream E protein targets that assist in this lineage choice are yet to be determined.

C. iNKT population expansion

In comparison to conventional T cells that undergo antigen-driven clonal expansion in the periphery,⁸¹ stage 1 iNKT cells start undergoing a major population expansion in the thymus immediately following positive selection and iNKT lineage commitment (**step 4**, figure 1).⁸² Several transcription factors, including NF- κ B^{34, 69} and c-Myc,^{70, 83} have been shown to be critical for proliferation of stage 1 and/or stage 2 iNKT cells. It should be noted that although PLZF expression is required for proper development of stage 1 and stage 2 NKTs, it doesn't seem to influence the proliferation capacity of these cells.⁷⁸ Our lab has demonstrated that high E protein levels achieved by deletion of both Id2 and Id3 led to a 3 fold increase in proliferation (and hence number) of stage 1 CD4⁺ iNKTs and DN TCR β ^{lo} type II NKTs.²⁵ As mentioned in the previous section, this increase in NKT cells is dependent on E protein levels as deletion of one copy each of E2A and HEB in Id2–Id3 deficient mice led to an expansion of $\gamma\delta$ T cells instead.²⁵ A similar increase in total NKTs has also been reported upon deletion of 3 out of 4 Id alleles.^{25, 26} Further, a mouse model with E2A and HEB deletion at the DP stage along with a V α 14 TCR transgene (to study roles of E proteins in an NKT selection-independent context) showed a 50% reduction in NKT numbers and a block beyond stage 0 partly due to defective proliferation in stage 1 cells.²⁶ It was also noted that there was an expansion of Id3 deficient NKT cells at stages 2 and 3 in Id3 deficient/wild type bone marrow chimeras, suggesting that Id3 limits iNKT expansion of not just stage 1, but at all stages of iNKT development. This increase in iNKT numbers was explained by increased proliferation in Id3 deficient cells, particularly at stage 2. Loss of Id2 however led to no change in iNKT proliferation.²⁶

The NKT expansion phase allows the lineage to make up for the limited initial selection and generate high copy numbers, which are important for quick response to antigens. It is interesting to note that both E protein and c-Myc have been shown to play a role in NKT proliferation.²⁶ However, it is unclear whether E proteins upregulate c-Myc or if they act in a concerted manner as observed in Burkitt's lymphoma.^{26, 84} Further, it has been suggested that E proteins may upregulate Id3 during this phase to keep a check on the proliferation.²⁶ Given that Id3 limits NKT expansion, it is yet to be determined if its expression needs to be reduced upon selection to increase NKT numbers,²⁶ and if so, how it is suppressed post TCR signaling. It has been suggested that the induction of the ITK/RAS/ERK (interleukin-2-inducible T-cell kinase/ Ras/ Extracellular-signal-regulated kinases) pathway, SLAM/SAP signaling or β -catenin may play a role in this.²⁷ It has also previously been demonstrated that Id2 can negatively regulate Id3 expression.^{26, 85} Thus, it is also possible that Id2

upregulation through cytokine signaling⁸⁵ then suppresses Id3 expression to allow proliferation.

Therefore, high E protein levels promote NKT population expansion during stages 1 and 2 of NKT development, while Id3 limits NKT cell numbers.

D. NKT differentiation and maturation

Similar to the T helper subsets, three NKT cell subsets NKT1, NKT2 and NKT17, have recently been characterized.⁷¹ The definition of conventional stage 2 NKTs has accordingly been revised to that of a heterogeneous population comprising of terminally differentiated NKT2 and NKT17 cells, and NKT1 progenitor cells⁷² (**step 5**, figure 1). These subsets later migrate to the periphery or stay in the thymus to mature (**step 6**, figure 1), and have varying transcriptional programs to direct distinct cytokine production in the periphery. While NKT1 cells are known for their IFN- γ production with high T-Bet (or T-box transcription factor, TBX21) and low PLZF expression, NKT2 cells are IL-4 and IL-13 producing with high GATA3 (GATA Binding Protein 3) and PLZF and moderate IL-17R β expression, and NKT17 cells are identified as IL-17 producing ROR γ ⁺ cells with low expression of PLZF and T-Bet.⁷¹ Similar to skewing of T helper responses in different mouse strains, the distribution of NKT subsets also varies widely between mouse strains.⁷² Due to the prevalence of NKT1 cells in C57BL/6J mice,^{86, 87} this subset has been extensively studied and is shown to mature in the thymus. Based on this, some consider mature NKT1 cells to be same as the conventional mature stage 3 NKTs²⁷ (NK1.1⁺ CD44⁺), but others believe that stage 3 can consist of all 3 mature subsets.²⁶ All three subsets localize to different locations in the periphery - NKT1 cells are found in the liver and spleen, NKT2 cells in the lung and NKT17 cells are found mostly in lymph nodes.⁷¹ The emergence of these subsets has not only changed the paradigm of a linear NKT developmental program, but has also enabled study of subset-specific roles of NKTs in disease contexts and expanded the potential applications of NKT cells as adjuvants to prime other cell types. Therefore, E and Id proteins play distinct roles in influencing the linear part of NKT development until stage 1, which includes lineage selection, commitment and expansion. Beyond stage 2, E and Id proteins play subset specific roles as differentiation into distinct NKT subsets requires unique transcriptional programs. The more recently discovered regulatory NKT (or NKT10) cells are not yet well characterized, and hence are not discussed here.^{88, 89}

For NKT1 development, both Id2 and Id3 have been demonstrated to be important as there is a significant reduction in the frequency of Id3-deficient T-bet⁺ NKT1 cells in Id3^{+/+}/Id3^{-/-} bone marrow chimeras, as well as reduced frequency and numbers of Id2-deficient NKT1 cells in Id2^{+/+}/Id2^{-/-} bone marrow chimeras.²⁶ This suggests that wild-type level of E protein activity (with both Id2 and Id3 present) supports NKT1 development, as observed in B6 mice. Further, the expression of T-bet, a critical transcription factor for NKT1 development as well as IFN- γ production and IL-2R β expression for stage 3 maturation,^{71, 90, 91} has also been deemed a critical checkpoint in NKT development.²⁷ It has been found that stage 2 iNKTs in ET-2 mice with sustained high E protein levels throughout NKT development failed to upregulate T-bet, causing a block in stage 3 maturation with impaired IFN- γ production.²⁸ These data suggest that E proteins can

directly or indirectly suppress T-bet expression to influence NKT maturation and effector functions. Id2 or Id3 deficiency in the BM chimeras described above also led to increased PLZF, but impaired T-bet expression, explaining the decrease in the NKT1 population.²⁶ Id3^{-/-} mice also have reduced numbers of stage 3 cells and IFN- γ producing iNKTs, along with significantly aberrant gene expression from as early as stages 1 and 2.²⁷ Based on the expression profile of Id3 (high in stages 1, 2 and NKT2 cells, but low in stage 3) and its requirement for the development of stage 3 NKT1 cells, the authors suggest that Id3 is important at earlier stages for initiation of the stage 3 maturation program. Deficiency of both Id2 and Id3 also leads to fewer IFN- γ only producing (i.e. NKT1) cells,²⁷ but it is still unclear whether Id2 and Id3 play distinct roles in the development of the subset, or the observations are merely artifacts of functional compensation by Id2 and Id3. Moreover, one of the early pieces of evidence suggesting that of Id or E proteins play a role in NKT development was the role of Id2 in maintaining the homeostasis of hepatic NKTs,²⁴ which are now known to be mostly NKT1 cells. These NKT1 cells were drastically reduced in Id2 deficient mice due to greater apoptosis caused by a reduction in CXCR6 (chemokine (C-X-C motif) receptor 6), Bcl-2 (B-cell lymphoma 2), Bcl-xL and the inability to counter the pro-apoptotic protein BIM, which is also an E protein target.

The role of Id3 in NKT2 development is ambiguous as Id3 deficiency led to a larger population of PLZF^{hi} NKT2 cells, but these cells also had the highest Id3 expression.²⁶ It is possible that the high level of Id3 is essential for selection into the NKT2 subset, but limits the numbers after selection by inhibiting E proteins that would otherwise drive increased differentiation into this subset. In support of this idea, ET-2 mice with sustained E protein expression had greater number of NKT2 cells.²⁸ Along similar lines, NKT17 cells are also promoted by high E protein levels, as reported by increase in this subset in ET-2 mice.²⁸ It is interesting to note that this subset has low to medium expression of both Id2 and Id3, as opposed to a dominant expression of one Id protein as seen in the other subsets.²⁶ Also, this increase in NKT2 and NKT17 cells was accompanied by a reduction in the NKT1 population, indicating that higher than normal E protein levels promote NKT2 and NKT17 subset differentiation at the expense of the otherwise predominant NKT1 lineage.²⁸ Moreover, retroviral overexpression of T-bet in immature NKTs from these mice rescued IFN- γ production and some other features of NKT1 cells, hinting at the plasticity of these subsets.

The discovery of these terminally differentiated subsets of NKT cells has altered our understanding of the development of this innate-like lineage. Although their key transcription factors and cytokine profiles have been well characterized, their differentiation from either a common or distinct NKT precursor(s) is not yet known. Further, it has been suggested that a separate checkpoint may exist for directing NKT cells towards NKT1, NKT2 or NKT17 fate, which is regulated by either transcription factors or TCR signal strength.²⁸ Since all three subsets localize to distinct locations in the periphery, it has been hypothesized that the cytokine milieu in the microenvironment of the NKT subset may also play a major role in their homeostasis.²⁶ Further, the plasticity of the subsets makes it even more difficult to uncouple the intrinsic transcriptional requirements from external stimuli. However, TCR signals and signal strength are unlikely to be the only external stimuli affecting the transcriptional program. The role of cytokines, chemokines and growth factors

in this setting are still waiting to be explored. It is interesting to note that besides influencing NKT differentiation, high E protein levels were also shown to impair thymic exit, causing a reduction in peripheral NKT cells.²⁸

All the above data suggest that E and Id protein levels can differentially influence the maturation and development of NKT subsets. High E protein levels were found to promote NKT2 and NKT17 development, whereas Id proteins were necessary for NKT1 development. The implications of these findings can be better understood by bearing in mind their resemblance to T helper subsets. For instance, E proteins are capable of directly regulating GATA3 expression,⁹² and Id2-deficiency in mice causes a Th2-dominant phenotype.⁹³ Id3 and E proteins have also been demonstrated to be critical for Th17 development by promoting ROR γ t expression and blocking IL-4 production.⁹⁴ Overall, these results indicate that E and Id proteins can regulate key transcription factors to bias NKT subset differentiation and their effector function.

III. DISCUSSION

In this review we have discussed the regulation of innate-like NKT cell development by E and Id proteins in detail. As mentioned before, there is evidence to suggest that E proteins can bind to and control PLZF expression, which is also a key transcription factor for other innate-like cells.²⁶ High E protein levels can promote both innate-like V γ 1.1V δ 6.3 $\gamma\delta$ T cells and iNKT cells.²⁵ Besides their common signature transcription factor PLZF, the innate-like V6.3 $\gamma\delta$ T cells and iNKT cells also have semi-invariant TCRs and very similar RNA expression profiles.⁹⁵ These observations suggest that these cells may independently diverge from mainstream $\alpha\beta/\gamma\delta$ lineages by acquiring an innate regulatory program. Alternatively, there may be a shared evolutionary history between these two innate-like lineages, which is distinct from conventional T cell development. A similar model of unique developmental pathways for innate-like and adaptive T cells has been proposed recently.⁶⁰ The authors found that unlike conventional $\alpha\beta$ and $\gamma\delta$ T cells, the development of innate-like $\gamma\delta$ and innate-like $\alpha\beta$ (NKT) cells is independent of the rearrangement status and expression of the TCR β and TCR γ chains respectively, suggesting that the innate-like T cell developmental program may diverge from conventional T cell development earlier than what is currently understood. In line with this, our evidence suggests that early deletion of Id proteins prior to selection at the DP stage may turn on an early NKT-specific program, causing a larger expansion of the NKT population.²⁵ Further, we found that the level of E proteins tightly regulate the lineage outcome between innate-like V γ 1.1V δ 6.3 and NKT cells. These data raise an interesting possibility that these innate-like cells may be evolutionarily ancient lineages with a shared developmental program that must be repressed by Id proteins to allow appropriate development and expansion of conventional $\alpha\beta$ T cells. It is important to note that this proposition doesn't challenge the role of CD1d-mediated selection in iNKT lineage specification at the DP stage, but rather speculates whether all DP cells have equal competence to become NKT cells or not.

Another challenge in this field is distinguishing between the unique and mutually compensatory roles of Id2 and Id3 in NKT development. Since Id2 deficiency doesn't lead to a noticeable phenotype but Id3 deficiency does,^{25, 27} it can be argued that Id3 can

compensate for Id2 but not vice versa. At the same time there is a more dramatic expansion in the NKT population upon deleting both Id2 and Id3.^{25, 27} While this phenotype can be explained solely based on higher E protein levels in Id2/Id3 double deficient mice than in Id3^{-/-} mice, it may also be mediated by a combined effect of distinct roles of Id2 and Id3 in NKT development. Based on the similarity between innate-like NKT cells and innate lymphoid cells (ILCs), which depend on Id2 for their development, it can be proposed that NKT cells also depend on Id2 but functional compensation by Id3 disguises any phenotype in Id2 deficient mice.²⁷ It is likely that Id proteins play compensatory or distinct roles depending on the stage of development and location. For example, it seems that Id3 is a key driver in the thymus whereas Id2 plays a more important role in the periphery.²⁷ Id2 and Id3 also seem to independently and uniquely regulate the development of different NKT subsets.²⁶ Moreover, we have found that Id2 is upregulated upon Id3 deletion in DN3 cells, but not in DP cells (unpublished data). Another group found, however, that Id2-deficient NKT cells possess higher Id3 expression while Id2 expression was reduced in Id3-deficient NKT cells.²⁶ This data suggests that Id2 attenuates Id3 expression, whereas Id3 supports Id2. These contradicting observations highlight the significance of the method (germline vs conditional knockout vs mixed bone marrow chimera) and stage of Id protein deletion in influencing NKT development. These factors should therefore be considered with care while inferring their role in NKT cells. Clearly, more work on the temporal and cross-regulatory aspects of E and Id proteins is needed to decode their myriad functions in NKT development.

To conclude, in this review we have highlighted several roles that E and Id proteins play at key “steps” in iNKT cell development. There has been some contradictory evidence regarding PLZF regulation and Id protein compensation which need to be investigated further. The early differentiation of NKT cells into the different subsets, and their regulation by E and Id proteins has also not been fully characterized. Due to the broad impact of these innate-like NKT cells on the immune response and disease outcomes, a better understanding of their development and regulatory mechanisms will facilitate manipulation of these cells in a clinical context.

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma-extra large
bHLH	Basic Helix Loop Helix
CD	Cluster of Differentiation
CXCR6	chemokine (C-X-C motif) receptor 6
DC	Dendritic Cell
DN	Double Negative
DP	Double Positive

EGR2	Early growth response 2
ERK	Extracellular-signal-regulated kinases
GATA3	GATA Binding Protein 3
HLH	Helix Loop Helix
Id	Inhibitor of DNA-binding
IFN	Interferon
IL	Interleukin
iNKT	invariant Natural Killer T
ILC	Innate lymphoid cells
ITK	interleukin-2-inducible T-cell kinase
Lck	lymphocyte-specific protein tyrosine kinase
MAIT	Mucosal Associated invariant T
MHC	Major Histocompatibility Complex
NK	Natural Killer
NKT	Natural Killer T
PLZF	Promyelocytic leukaemia zinc finger protein
RAG1/ RAG2	Recombination activating gene 1/2
RORγt	RAR-related orphan receptor gamma T
SAP	signaling lymphocytic activation molecule (SLAM)-associated protein
SLAM	Signaling lymphocytic activation molecule
SP	Single Positive
TCR	T cell Receptor

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FIGURE 1. Stages of NKT cell development and their regulation by E and Id proteins

According to the currently accepted model, NKT cell development is concurrent with conventional $\alpha\beta$ T cell development up to the DP stage. There are several important “steps” (depicted as numbered diamonds) in NKT development that are critical for their appropriate lineage specification and functional maturation, and are regulated by E and Id proteins to influence their development. A functional TCR β rearrangement allows DN3 cells to proceed beyond pre-TCR selection (step 1) and reach the DP stage where α rearrangement takes place. The DP cells that then stochastically express a Va14Ja18TCR (step 2) typically diverge from conventional $\alpha\beta$ T cells upon CD1d-mediated selection and undergo iNKT lineage commitment marked by PLZF expression at stage 0 and 1 (step 3). This is followed by an expansion phase at stage 1 (step 4) and terminal differentiation into iNKT1, iNKT2 or iNKT17 stage 2 subsets (step 5). These subsets with varying cytokine profiles, then migrate to different parts of the periphery to mature (step 6). The iNKT cells that mature within the thymus were previously characterized as stage3 iNKTs (step 6), but have now been identified to be iNKT1 cells. The regulation of these critical NKT developmental steps by E and Id proteins is summarized in Table1. E and Id protein expression during NKT cell development (beyond DN3 stage) has also been graphically represented below the corresponding stages (dark red represents highest expression, white represents little to no expression).

TABLE 1
Role of E and Id proteins in iNKT development

E and Id proteins have recently been shown to play several roles in NKT TCR rearrangement, NKT lineage commitment, expansion, differentiation and maturation. Thus E and Id proteins regulate this lineage at several key NKT developmental steps which are marked in figure 1. Their functions at the different stages of NKT development are summarized in this table.

No.	Developmental step	E or Id protein role
1	Pre-TCR / β selection : DN3b stage	<ul style="list-style-type: none"> High E proteins suppress selection²⁵
2	iNKT TCR rearrangement/ iNKT selection : DP stage	<ul style="list-style-type: none"> HEB promotes secondary rearrangement through increased survival²³ Id3 prevents secondary rearrangement by turning off <i>rag</i>²⁷ Id2 and Id3 prevent early Vα14-Jα18 rearrangement²⁵
3	iNKT program initiation : iNKT Stage 0	<ul style="list-style-type: none"> E proteins bind to PLZF directly and promote expression²⁶ Id protein deficiency lead to lower PLZF²⁷
4	iNKT proliferation : mainly Stage 1	<ul style="list-style-type: none"> Id2 and Id3 limit iNKT proliferation²⁵ Id3 limits iNKT numbers through all development stages²⁶
5	iNKT subset differentiation: Stage 2	<ul style="list-style-type: none"> NKT1 development needs Id2 and Id3; NKT2 cells expand on Id3 deficiency²⁶ NKT2 and NKT17 are supported by high E protein (or low Id proteins)²⁸
6	iNKT Stage 3 maturation	<ul style="list-style-type: none"> Id3 needed at Stages 1 and 2 for initiating appropriate Stage 3 program²⁷ E proteins prevent T-bet expression which is needed for maturation²⁶