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The divergence between T cell and innate lymphoid cell fates controlled by E and Id proteins

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T cells develop in the thymus from lymphoid primed multipotent progenitors or common lymphoid progenitors into αβ and γδ subsets. The basic helix-loop-helix transcription factors, E proteins, play pivotal roles at multiple stages from T cell commitment to maturation. Inhibitors of E proteins, Id2 and Id3, also regulate T cell development while promoting ILC differentiation. Recent findings suggest that the thymus can also produce innate lymphoid cells (ILCs). In this review, we present current findings that suggest the balance between E and Id proteins is likely to be critical for controlling the bifurcation of T cell and ILC fates at early stages of T cell development.

KEYWORDS

E2A, HEB, innate lymphoid cells, Id2, Id3, T cells

Introduction

The E protein family of transcription factors are crucial molecules engaging in B cell development in the bone marrow and T cells differentiation in the thymus (1, 2). This family consists of proteins encoded by three genes, E2A (also called *Tcf3*), HEB (*Tcf12*) and E2-2 (*Tcf4*) (Figure 1A) (3–5). These proteins share extensive sequence homologies in the activation domains (AD1, LH) and basic helix-loop-helix (bHLH) DNA-binding domain (6–9). E proteins regulate the transcription of their target genes by forming homodimers or heterodimers and bind to E-box sequences (9). The E2A gene gives rise to two proteins, E12 and E47, due to alternative splicing of two adjacent exons, each encoding a basic helix-loop-helix (bHLH) domain (10). While E47 binds DNA avidly as homodimers, E12 does so poorly due to the presence of an inhibitory domain (11). However, both form heterodimers with other bHLH proteins such as MyoD, and bind DNA efficiently. The HEB gene encodes a full-length canonical protein (HEBCan) and a truncated alternate form (HEBAlt), which derives from a transcript initiated in the middle of the gene (12). HEBAlt lacks the AD1 transcription activation domain and has lower transcriptional activities (13). It has an Alt domain at the N-terminus with three

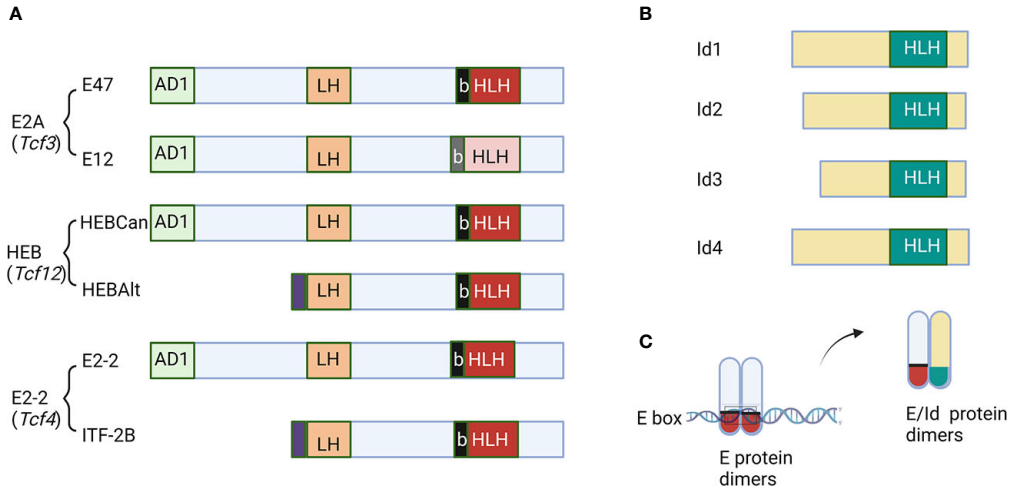

FIGURE 1
Schematic diagrams of E and Id proteins. **(A)** The function domains of E proteins are labeled. AD1 and LH are two transcription activation domains. The basic and helix-loop-helix domains are marked as b and HLH, respectively. **(B)** Id proteins with the HLH domain are shown. **(C)** The mechanism of action of Id proteins to inhibit DNA binding by E proteins. The figure was created by BioRender.com.

tyrosine residues which can be modified by phosphorylation that augments its transcriptional activity (13).

The family of inhibitor of differentiation proteins, Id1-4, antagonize E proteins by dimerizing with them *via* the helix-loop-helix domain (Figure 1B) (14–19). However, because Id proteins lack the basic amino acids necessary for DNA binding, heterodimers between E and Id proteins cannot bind to E box sequences (Figure 1C). Transcription of the E protein genes is less variable but that of the Id genes is highly dynamic. Therefore, the net E protein activity in a given cell is determined by the levels of both E and Id proteins (16, 17). In this review, we intend to highlight the roles of E and Id proteins in regulating the fate choices between T cells and innate lymphoid cells.

T cell development

Lymphoid-primed multipotent progenitors (LMPP) and common lymphoid progenitors (CLP) travel from the bone marrow to the thymus and become early T cell progenitors (ETP) (20–23). T cell developmental progression in the thymus can be generally defined by the expression of CD4 and CD8 surface markers: from CD4 and CD8 double negative (DN) to double positive (DP) and then to CD4 or CD8 single positive (SP) (24–27). During the transition from DN to DP stage, an immature CD8 single positive subset (ISP) has been described (28, 29). Within the DN compartment, four subsets (DN1 to DN4) are characterized by the expression of c-kit and CD25 in the order of maturity as c-kit$^+$CD25$^-$, c-kit$^+$CD25$^+$, c-kit$^-$CD25$^+$ and c-kit$^-$CD25$^-$ (27). ETPs are at the top of the hierarchy and included in the DN1 subset (23). They give rise to both αβ and γδ T cells, which have distinct T cell receptors (TCRs), different developmental programs and divergent functions. The E2A and HEB genes are both expressed in the thymus. Interestingly, HEBAlt is preferentially produced in the DN and ISP stages. Since E proteins are known to inhibit cell proliferation and HEBAlt acts as a hypomorph (13, 30), whether HEBAlt plays a role in tampering E protein activities during pre-TCR-triggered cell expansion is interesting to be investigated.

αβ T cells

The development of αβ T cells is largely driven by αβ TCR signaling events. However, before the formation of pre-TCRs and TCRs, the differentiation of committed T cell precursors is supported by Notch signaling and signaling from cytokine receptors such as that of IL-7 (31–35). Critical transcription factors involved in T cell commitment include TCF1, GATA3 and Bcl11b (36–40). The sequential rearrangements of TCRβ and then TCRα genes catalyzed by the RAG1 and RAG2 recombinases set the milestones of the developmental progression (24, 41–44). The TCRβ locus undergoes recombination between D to J regions and then V to DJ regions to produce functional β chains, which pair

with the pre-Tα (45, 46). The pre-TCR complex delivers signals leading to the expansion of DN3 cells and their advancement to the DP stage. The TCRα gene rearrangement occurs at the DP stage, which allows the formation of αβ TCRs, triggers the positive and negative selection and enables the generation of mature SP T cells (47–49). Mature but naive T cells leave the thymus by the upregulation of S1PR1 and CD62L (50–53).

αβ T cells possess a large repertoire of TCRs due to a collection of V regions. These TCRs recognize diverse antigens presented by the MHC molecules and elicit subsequent signaling events. $CD4^+$ and $CD8^+$ naïve T cells exit the thymus to be activated and differentiate into helper and cytotoxic effectors in peripheral lymphoid organs, respectively (54, 55). Due to the sheer quantity of thymic output of αβ T cells and their ability to proliferate in response to antigen engagement, αβ T cells are the major players of adaptive T cell immunity.

γδ T cells

The development of γδ T cells differs from αβ T cells. Firstly, unlike αβ T cells, γδ T cells do not traverse DP and SP stages during the development. Instead, they undergo γδ lineage commitment and maturation at DN2 and DN3 stages (56–58). Generation of mature γδ T cells depends on the V-J rearrangement of the TCRγ locus and V(D)J recombination in the TCRδ locus, along with Notch signaling. Since the TCRδ gene is embedded in the TCRα locus, TCRα rearrangement, triggered by pre-TCR signaling after an independent rearrangement event of the TCRβ gene, can eliminate the TCRδ gene, thus aborting the γδ T cell fate (59–61). Early precursors of effector γδ T cells in the thymus are identified as $CD24^+$ and then mature to $CD24^-$ stage (62, 63). There are three types of γδ T cells classified based on their effector functions, γδ1, γδ17 and innate-like γδ T cells, which secrete interferon γ, IL-17 and interferon γ plus IL-4, respectively (64–66). The development of γδ T cells require stronger TCR signals in the comparison to αβ T cells (67, 68). The gradients of TCR signals determines the development of specific effector subsets. The generation of innate-like γδ T cells depends on the strongest TCR signal as indicated by their higher levels of CD5 compared to other γδ subsets (69). CD5 levels are proportional to TCR signaling strength in the thymus (70, 71). Expression of PLZF transcription factor also depends on ligand ligation with TCR and PLZF is required for the effector function of innate-like γδ T cells (72). Type 1 γδ T cells also require a strong TCR signal and the T-bet transcription factor is critical for γδ1 differentiation (65, 72–75). On the other hand, type 17 γδ T cells rely on a weaker TCR signal for the differentiation (65, 72, 74, 76). In fetal organ culture, addition of activating antibodies against γδ TCR or CD3 impairs the production of γδ17 cells (76). Moreover, RORγt transcription factor is essential for γδ17 development (77). Additionally, CD73 expression marks most of γδ T cells committed to mature into effector cells in the thymus (78).

Distinct subsets of γδ T cells reside in different tissues and develop at different ages in mice (56, 57). The Vγ regions are described by two different nomenclatures. In this review, we will use the one defined by Raulet and colleagues (79). In the early fetal stage, the first wave of γδ T cells is associated with the $V\gamma3^+V\delta1^+$ subset known as the dendritic epidermal cells, which produce IFNγ (65, 80, 81). The development of the $V\gamma4^+$ subset begins at the fetal stage and lasts until birth. The generation of the $V\gamma2^+$ subset occurs in the late fetal stage and continues through adulthood. The $V\gamma2^+$ subset consists of cells producing IL-17 or IFNγ (82). The IL-17-producing cells become long-lived cells with self-renewal capabilities after birth (83). $V\gamma1.1^+$ cells develop at the prenatal stage and this persists through adult life (56). Despite the complicated developmental schemes of γδ T cell differentiation, how γδ TCRs interact with their ligands and elicit signals is less understood. To some extent, γδ T cells are thought to have properties resembling innate cells.

T cell development is a "wasteful' process". Every D-J or V-DJ combination only has one third of a chance to create an in-frame joint that result in a full-length TCR chain. It is believed that over 70% of the developing T cells do not reach the mature stage and die because they fail to form pre-TCR (β selection) at the DN3 stage or because they cannot produce a full-length TCRα chain at the DP stage (death by neglect). They can also be eliminated due to excessively strong TCR signaling (negative selection). Are there any alternative fates for these T cell "drop-outs"? Perhaps, innate lymphoid cells are some of the options.

Regulation of T cell development by E and Id proteins

E proteins play pivotal roles in governing the development of αβ T cells. Two of the E protein genes, E2A and EBCan, are expressed in T cells and they have redundant functions. The proteins encoded by these two genes include E12, E47, HEBCan and HEBAlt. Since all knock-out constructs targeted the bHLH domains, E2A or HEB deficient mice lack all of their respective proteins. Germ-line ablation of either E2A or HEB gene partially impairs T cell development by dramatically reducing thymocyte counts (84, 85). The leaky block allows the maturation of small numbers of T cells, which are predisposed to develop T cell lymphoma (84–86). HEB deficiency also reveals a novel role of HEB at the ISP stage (86). In contrast, simultaneous inhibition of all E proteins by expressing Id1 using the proximal promoter of *lck* in transgenic mice results in a complete block of T cell development, arresting thymocytes at the DN1 stage when the Id1 transgene begins to be expressed (87, 88). Likewise, inducible ablation of both E2A and HEB genes using the *plck-Cre*

transgene results in a developmental arrest at the DN3 stage when the Cre gene is expressed (89).

E protein-mediated control at these early stages of T cell development is multi-dimensional. First, E proteins are known to activate the transcription of *Notch1*, which encodes the receptor for Notch ligands such as Delta-like 4 in the thymus and ensure the differentiation and survival of T cells (90–92). Second, E proteins are found to activate the transcription of *Rag1* and *Rag2* (93, 94), which code for the enzymes essential for VDJ recombination of TCR genes. Third, E proteins facilitate TCR gene rearrangement by increasing chromatin accessibility at the TCRβ locus (95). Fourth, the binding of E2A-HEB heterodimers to *Ptcra* enhancer regulates pre-Tα expression at the DN3 stage (96–98). Finally, the interplay between E proteins and other transcription factors such as TCF1 and LEF1 also contribute to the positive regulation of early T cell development (36, 99).

Following pre-TCR signaling, the Ras-MAP kinase pathway is activated, which leads to the up-regulation of Egr transcription factors and then activation of the Id3 gene (100–103). This suggests that down-regulation of E protein activity is necessary for DN3 cells progress to the DP stage. Indeed, when *Rag1* was deleted, T cell development arrested at the DN3 stage (104). However, if E proteins are down-regulated by germline E2A deletion or *pLck-Id1* expression, *Rag1*$^{-/-}$ thymocytes can advance to the DP stage (105, 106). Another mechanism to down-regulate E proteins is to accelerate their ubiquitin-mediated degradation in the presence of Notch signals and MAP kinases activated by pre-TCR signaling (107, 108).

At the DP stage, Id3 expression is transiently triggered by TCR signaling and is involved in the positive selection of developing thymocytes (101, 109). Deleting both Id2 and Id3 genes prevented the progression of positively selected T cells to the SP stage (110). Conversely, low levels of Id1 expression in *plck-Id1* heterozygous transgenic mice allows some T cell precursors reach the DP stage but a majority of these cells undergo apoptosis likely due to excessive responses to the normal levels of TCR stimulation (105, 111). This notion was supported by the observation of hyper-activation of NFκB upon ectopic Id1 expression (105, 112). In addition, deleting both E2A and HEB genes also impairs the generation of CD4 SP T cells (110). Collectively, E and Id proteins clearly are the central players in shaping αβ T cell development.

A strong TCR signal triggers the activation of the ERK-Egr-Id3 axis and favors γδ over αβ T cell development (73). Id3 deficiency resulted in an expansion of Vγ1.1^{+} innate-like γδ T cells, possibly due to the dampening of the strong TCR signaling which normally causes the death of these cells (113, 114). In fetal organ cultures, HEB deficiency impairs the differentiation of Vγ4 and Vγ6-containing γδ17 cells. In *et al.* postulated two pathways of γδ T cell development (115). Pathway 1, which favors γδ1 cells, depends on strong TCR signaling and up-regulation of Id3. In contrast, pathway 2 mostly occurs in the fetal stage and requires lower levels of TCR signaling and Id3 expression. HEB is necessary for Vγ6^{+}CD73^{-} γδ17 T cells in the fetal stage as well as Vγ4^{+}CD73^{+} γδ17 T cells in neonates (115). HEB and E2A are thought to activate the transcription of *Sox4, Sox13* and *Rorc* genes necessary for γδ17 differentiation (115, 116). Overall, it appears that Id3 expression plays a critical role in directing γδ T cell development through counterbalancing the function of E proteins.

Differentiation of innate lymphoid cells

Innate lymphoid cells (ILCs) are first responders in immune reactions towards environmental insults and microbial infections. ILCs are divided into three groups, ILC1 to ILC3, which play different roles during specific immune responses (117, 118). Even though ILCs share with T cells the transcriptional factors that drive their differentiation and the profiles of cytokine production, they lack T-cell receptors (TCR), thus eliciting innate immunity as opposed to adaptive immunity mediated by T cells (118–120). Each ILC subset has been increasingly recognized to be heterogenous and display different characteristics in different tissues (121). Plasticity between the three ILC subsets also exist, especially under pathophysiological conditions (118, 122). Nevertheless, the general properties and functions of these three subsets of ILCs have been established. The ILC1 group consists of helper-like ILC1s and conventional NK cells (cNK). ILC1s mediate the early immune response upon contact with intracellular pathogens like bacteria and viruses. Their effector function regarding cytokine production is similar to the that of cNK cells, namely secreting IFNγ upon pathogen exposure. However, NK cells but not helper-like ILC1s are cytotoxic and able to produce high levels of cytotoxic granules like perforin and granzymes. The T-bet transcription factor is responsible for ILC1 differentiation and function (123). ILC2s share a transcriptional network and cytokine production profiles with those of type 2 T helper cells (Th2). GATA3 is the signature transcription factor and drives the expression of cytokines including IL-5, IL-13, IL-4, IL-9, and amphiregulin (124–126). RORα is another transcription factor indispensable for ILC2 differentiation (127). ILC2s are crucial for the protection against helminth infection. They are also activated by allergens due to the release of IL-25, IL-33 and TSLP in the tissues, contributing to a number of respiratory diseases such as asthma (128). On the other hand, ILC2s have also been shown to be involved in tissue repair following influenza infection (129). The ILC3 group includes innate immune cells committed to targeting extracellular microbes. They reside mainly in the mucosal tissues and maintain their homeostasis locally. ILC3s express RORγt and produce cytokines such as IL-17A, IL-22, and GM-CSF (118, 123). Lymphoid tissue inducers (LTis) are a subset of ILC3s essential during the fetal stage for

supporting the development of lymph nodes and other lymphoid tissues (130).

Innate lymphoid cells are progenies of hematopoietic stem cells, arising from progenitors destined to become lymphoid cells such as lymphoid-primed multipotent progenitors (LMPPs) or common lymphoid progenitors (CLPs) (20, 21). These progenitors reside in fetal liver or adult bone marrow where ILCs differentiate in addition to B cells. These processes have been extensively studied as summarized below. However, LMPPs and CLPs also travel to the thymus to produce T cells. The capability of the thymus to support ILC differentiation has recently become appreciated (90, 131–133). The divergence of T cell development to ILC fates is an interesting issue to be addressed here. Finally, ILCs are also believed to be derived from tissue-resident progenitors but at what stage these progenitors seed the peripheral tissues and whether all ILC subsets utilizes this mechanism of reproduction are not fully understood.

ILC differentiation in the bone marrow and fetal liver

Innate lymphoid cells develop in the bone marrow from LMPPs or CLPs through a series of intermediate progenitors which progressively lose the potential of giving rise to B cells and then NK cells (120). The progenitors that can generate subsequent progenitors for either ILC or NK cells are called alpha LPs (αLPs), which require the NFIL3 and TOX transcription factors (134). Early innate lymphoid progenitors (EILP) characterized by TCF1 expression, also have a similar differentiation potential (135). Next, common helper ILC progenitors (CHILPs) are regulated by Id2 and responsible for the ILC but not NK subsets (136). ILC progenitors (ILCPs) controlled by PLZF are dedicated to only producing ILCs, and are found in both bone marrow and fetal liver (137). In contrast, NK progenitors (NKPs) which also express Id2 are specialized to become NK cells (120, 137). Although CHILPs or ILCPs have the potential to give rise to all three ILC subsets *in vitro* when cultured on OP9-DL1 stroma, the predominant subset detected in the bone marrow is ILC2 as well as their precursors called ILC2Ps (138). Moreover, there is also evidence that ILC1s can be generated in adult liver from fetal hematopoietic stem cells (139).

Whether the bone marrow serves as a constant source of ILC2 replenishment has not been well established. Experiments using parabionts suggested tissues such as the lung receive few ILC2s from the blood circulation (140, 141). However, recent single cell RNA sequencing (scRNAseq) data showed a population of ILC2s in the blood of wild type and athymic nude mice, which suggest that these ILC2s may come from the bone marrow or they are the recirculating ILC2s from peripheral tissues (133). IL-18R$^+$ precursors of ILC2s have also been found in the lung and shown to arrive from the blood (142, 143).

In humans, ILC progenitors with biases to different ILC subsets are readily detectable in the blood (144, 145). Likewise, committed ILC1 to ILC3 subsets are also found in the blood (122, 146). These cells are assumed to come from the bone marrow but no direct evidence is available. The frequencies of the ILC subsets are often found to be altered in different disease states, which may potentially serve as biomarkers of these diseases (147–149).

ILC differentiation in the thymus

Small numbers of ILCs, particularly ILC2s, have been found in the thymus at pre- and post-natal stages (150–154). This is consistent with the fact T cell progenitors express the transcription factors supporting ILC2 differentiation, namely GATA3, TCF1 and Bcl11b (155).

Whether the thymus is another lymphoid organ capable of exporting ILC precursors or ILCs to peripheral tissues was investigated by using scRNAseq of the lineage negative (Lin$^-$) Thy1$^+$ fraction of the blood of wild type and athymic nude mice (133). Bajana *et al.* found that about half of the ILC-containing Lin$^-$Thy1$^+$ population, was greatly diminished in the athymic nude mice, which suggest that the production of these cells is thymus-dependent, thus designated td-ILCs. These cells were fractionated into four clusters based on their distinct transcriptomic properties. All td-ILCs express genes commonly expressed in ILCs such as *Tcf7* and *Il7r* but they lack the signature transcription factors that specify ILC1 to ILC3: T-bet, GATA3 and RORγt, suggesting that td-ILCs can be ILC precursors. Indeed, when these cells were isolated as Lin$^-$Thy1$^+$CD127$^+$CD62L$^+$ from the blood and cultured on OP9-DL1 stroma, different subsets of ILCs were generated (133). Whether this population contains disparate progenitors for distinct ILC subsets or progenitors with multiple potentials is to be determined.

Interestingly, td-ILCs express *Cd3d*, *Cd3e* and *Cd3g* but no other T cell specific genes such as *Cd4, Cd8a, Rag1, Rag2* and *Dntt*. Flow cytometry analyses detected CD3ε by intracellular staining but not by surface staining (133). Moreover, td-ILCs do not have TCRβ or TCRδ either on the surface or in the cytoplasm, thus indicating that they are not T cells. Using intracellular CD3ε (icCD3ε) as a marker, Bajana *et al.* also detected icCD3ε$^+$ cells in the lung, small intestine and skin of wild type mice (133). Because these icCD3ε $^+$ cells are greatly diminished in nude mice, the results were interpreted to mean that icCD3ε marks thymus-derived cells. Like in blood td-ILCs, the icCD3ε$^+$ cells in the lung and small intestine do not express TCRβ or TCRδ, ruling out the possibility that they are T cells. This suggests that td-ILCs in the blood may home to peripheral tissues where they differentiate into diverse ILC subsets. In the lung, a significant fraction of icCD3ε$^+$ ILCs are ST2$^-$RORγt$^+$ ILC3-like cells. In contrast, the lamina

propria of small intestine harbors icCD3ϵ^{+}KLRG1^{-}T-bet^{+} ILC1-like cells. Curiously, the expression levels of GATA3 correlated inversely with those of icCD3ϵ, which suggests that ILC2 differentiation is accompanied by the down-regulation of CD3 expression (133). Although this possibility remains to be investigated, the potential down-regulation of CD3 expression makes it difficult to assess the contribution of thymus-derived ILC2s to the overall ILC2 pool. A lineage-tracing system with a *Cre* transgene that is specifically and efficiently expressed at the early stages of T cell development would greatly facilitate the estimation of the contribution of thymus-derived ILC2s and further validate the thymic origin of ILC2 subsets.

Additional evidence exist that support the notion that the thymus contributes to the ILC2 pools. Qian *et al.* showed that not only multipotent progenitors (DN1) but also committed T lineage cells (DN3) from the thymus can differentiate into functional ILC2 on OP9-DL1 stromal cells (132). Consistently, ILC2s isolated from the lung of WT but not nude mice harbor rearranged TCR genes, *Tcrb* and *Tcrg*, suggesting that at least some of the ILC2s originated from committed T lineage cells in the thymus (132, 156). While *Tcrg* rearrangement was readily detectable by electrophoresis, analyses of the D-J and V-DJ recombination in the *Tcrb* locus required Southern blotting because of the diversity of their rearrangement events. Shin *et al.* sequenced the rearranged *Tcrg* segments and found a reduced frequency of in-frame rearrangement in ILC2s compared to that in γδ T cells (156). It was thus concluded that ILC2s are derived from cells which have failed productive γδ TCR rearrangement (156, 157). However, further investigation at the single-cell level could strengthen the conclusion. Despite the rearrangement events detected, ILC2s do not express TCRβ or TCRδ either intracellularly or on the surface.

Likewise, NK cells have also been shown to arise from early T cell precursors in the thymus, suggesting a branch point between T and NK cells (158–160). It remains to be determined if this branch point is similar or different from those giving rise to ILCs.

Regulation of ILC differentiation by E and Id proteins

Id2 is expressed in ILC progenitors and plays an essential role in ILC development, which implicates the involvement of E proteins in regulating ILC differentiation (136, 161). Strikingly, down-regulation of E proteins by the ectopic expression of Id1 in transgenic thymocytes at the DN1 stage or by deletion of the E2A and HEB genes with *plck-Cre* at the DN3 stage led to dramatic increases in ILC2 production in the thymus (131, 132). As a result, large amounts of ILC2s were exported from the thymus to peripheral tissues throughout the body. The thymus was shown to be responsible for the mass production of ILC2 in Id1 transgenic mice because when the transgene was bred onto the nude background, ILC2 expansion was no longer detectable (132). ILC2s made in the thymus of Id1 transgenic and E protein deficient mice respond to IL-25 or IL-33 stimulation similarly as wild type ILC2s by secreting IL-5 and IL-13 in cultures (131, 132). *In vivo*, Id1 transgenic mice exhibited greater type 2 responses when treated with papain in the lung or during helminth infection (131). These are likely due to the presence of excessive amounts of ILC2s in Id1 transgenic mice. However, on a per cell basis, Id1 transgenic ILC2s appeared to have a less robust production of IL5 and IL-13 (131). It is not clear if this is due to a cell intrinsic difference or a limitation of stimuli available to all of the extra ILC2s in Id1 transgenic mice. Barshad *et al.* made a similar observation by treating wild type and Id1 transgenic mice with house dust mites (HDM) (162). By analyzing the chromatin accessibility, they found a reduction in AP-1 and C/EBP binding sites in open chromatins after HDM treatment in Id1 transgenic ILC2s. Whether this is due to a direct or indirect effect of E protein inhibition remained to be determined.

In the blood of *Tcf3*$^{fl/fl}$*Tcf12*$^{fl/fl}$*plck-Cre* mice, an extremely large population of cells (cluster 0) that belong to thymus-dependent ILC precursors was detected using scRNAseq (133). In addition, a subset (cluster 2) with characteristics of NK cells was also markedly enriched (133). These cells can give rise to different ILC/NK subsets when cultured on OP9-DL1 stroma (133). Together, these results suggest that E proteins play multiple roles in suppressing the production of ILC and NK precursors, which may arise at different developmental stages or from different T cell precursors. Whether E proteins suppress the same or different transcriptional programs governing ILC and NK differentiation remains to be investigated.

Ablating E2A and HEB genes starting at the CLP stage using *IL7r-Cre* increased the production of both ILC2s and LTi-like cells, a subset of ILC3s (90). Conversely, inducible expression of a gain-of-function mutant of E47 by *Rag1-Cre* impaired the differentiation of ILC2s from ILCP in the bone marrow (163). Furthermore, Id2$^{-/-}$ mice have been shown to be devoid of NK cells and lymph nodes which are initiated by LTi cells (130). Yet, overexpression of Id3 in human hematopoietic stem cells promoted NK differentiation (164). These findings suggest that down-regulating E protein function is instrumental for NK cell differentiation (165). It was further shown that Id2 plays a key role in regulating the production of IL-15 important for NK homeostasis (166, 167).

Transcriptional programs of E protein-mediated suppression of ILC differentiation

Inducible deletion of the E2A and HEB genes promoted ILC2 differentiation from CLP, DN1 and DN3 cells on OP9-DL1

stroma by 20-40 folds, which demonstrates a powerful cell-intrinsic suppression by E proteins (132). It is therefore interesting to elucidate the transcriptional programs that underlie the suppression of ILC2 differentiation. Miyazaki *et al.* performed RNA sequencing and Assay for Transposase-accessible Chromatin Sequencing (ATAC-seq) using DN1 (ETP) cells of fetal thymi of control and *Tcf3*$^{fl/fl}$*Tcf12*$^{fl/fl}$*Il7r-Cre* mice. As expected, they found the down-regulation of an array of genes important for T cell development, which include *Notch1, Ptcra, Rag1, Rag2* and *Cd3d*. On the other hand, genes known to be expressed in ILC progenitors or ILC2s were up-regulated. Examples of such genes are *Pdcd1, Il18r, Id2, Gata3, Lmo4, Rora, Tox, Est1, Il4, Il1rl1* and *Klrg1*. The chromatin accessibility assays also showed a shift from the open chromatin patterns of T cells to those of ILCs. While these findings agree with the phenotypes of E protein deficient mice, it is difficult to pinpoint the critical switches that alter the cell fates.

Likewise, Qian *et al.* conducted RNA sequencing using DN1 or DN3 cells from control and *Tcf3*$^{fl/fl}$*Tcf12*$^{fl/fl}$*Rosa26*^{CreERT2} mice cultured on OP9-DL1 stromal cells (132). On day 4 of the culture, tamoxifen was added to the medium and the cells were collected 24 or 72 hours later. Expression of genes important for T cell development decreased whereas those crucial for ILC2 differentiation increased. Even after one day of E-protein ablation, a collection of genes coding for diverse transcription factors became activated. These include *Zbtb16, Gata3, Rora, Rxra, Klf6, Ikzf2* and *Irf4*. While it is possible that E proteins individually repress the transcription of all of these genes, a coordinated program that controls the transcription of critical factors essential for ILC2 differentiation may be at play.

A close-up look at the action of E proteins was carried out by making use of the E47-ER fusion proteins (112), which allowed instant induction of E protein activity upon addition of tamoxifen (168). ILC2s from the thymus of Id1 transgenic mice were transduced with retroviruses expressing E47-ER or empty control viruses. Transduced cells were isolated by sorting for EGFP expressed off the same retroviral vector. After expansion, these cells were then incubated with tamoxifen for 4 or 16 hours and harvested for RNA sequencing or ATAC-seq. Consistent with the function of E proteins as transcription activators, Peng *et al.* found more genes activated than repressed by E47-ER at both time points (168). Among them are three genes encoding transcriptional repressors, *Cbfa2t3, Jdp2 and Bach2* (169–171).

Interestingly, ATAC-seq data showed that a modest increase in chromatin accessibility 4 hours post induction of E47 was followed by a widespread reduction in open chromatin regions 16 hours later. Moreover, the transcription factor motifs enriched in the differential peaks shifted from those bound by bHLH and Ets1 proteins at 4 hours to those recognized by bZip and GATA factors. It is therefore possible that one of the mechanisms whereby E proteins suppress ILC2 differentiation is to control the expression of transcription repressors, which in turn negatively regulate the transcription of genes important for ILC2 differentiation or function. Although this hypothesis has not been validated through genetic complementation studies, the correlation between the alteration of gene expression in *Cbfa2t3*$^{-/-}$ and E protein deficient mice support this idea (168, 172). Proteins bound to bZip and GATA motifs such as Batf and GATA3 are also known to be crucial for ILC2 function (126, 173, 174).

The RORα transcription factor also plays an important role in ILC2 differentiation (127). *Rora*$^{-/-}$ mice lack ILC2s but have intact T cell compartments. Recently, Ferreira *et al.* showed that RORα promotes ILC2 over T cell development by activating the transcription of *Id2* and *Nfil3*, which in turn inhibit the function of E proteins (153). However, in E protein deficient thymocytes, *Rora* expression is consistently up-regulated (90, 132, 168). Thus, a positive feedback loop may perpetually cause the up-regulation of *Rora* expression during ILC2 differentiation. There are likely additional transcription factors which act in parallel or in sequence to coordinate the differentiation of ILC2s and possibly ILC progenitors. However, it is clear that E proteins and their inhibitors, Id proteins, play a central role in maintaining the balance between T cell and ILC development.

The crossroads of T cells and innate lymphoid cells

The major difference between T cells and innate lymphoid cells is the presence and absence of TCRs on their cell surface, respectively. However, there are a number of common features in the differentiation of these two types of cells (175). The thymic environment is conducive to the maturation of both T cells and ILCs (at least ILC2s and ILC3s) by supporting Notch and IL-7 signaling. The thymic progenitors equipped with transcription factors such as TCF1 and GATA3, are able to differentiate into both T cells and ILCs. Obviously, T cell production is the dominating responsibility of the thymus. This is due to the overwhelming effects of TCR-driven T cell expansion and powerful transcriptional programs in place to ensure an adequate T cell output. One of such transcriptional programs is controlled by the balance between E and Id proteins (Figure 2). When E protein activities are high, T cell development proceeds. When Id proteins overcome E proteins, ILCs can develop. Although Id2 has been shown to be expressed in ILC progenitors and play critical roles in ILC differentiation in the bone marrow, expression of Id3 is stimulated by TCR signaling in both αβ and γδ T cells (73, 106). This would create opportunities for developing T cells to divert to the ILC path. However, this possibility needs to be vigorously investigated. It is also interesting to explore whether the large numbers of developing T cells eliminated during the differentiation processes could be recycled into ILCs and used to replenish ILC pools in peripheral tissues. The E/Id axis has clearly been

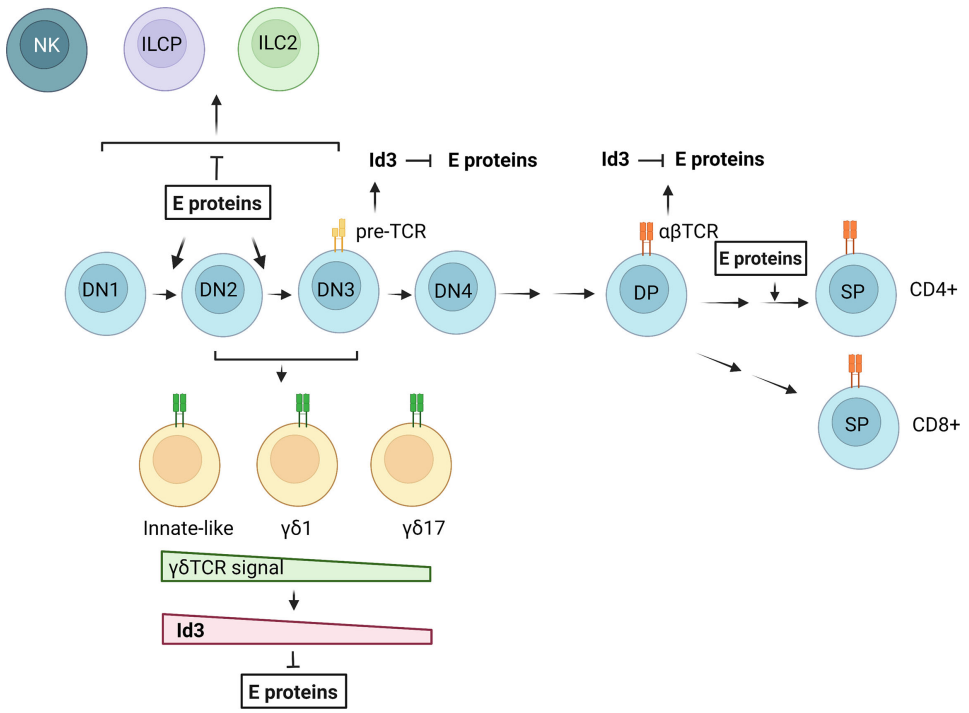

FIGURE 2
Regulation of T cell and ILC differentiation by E and Id proteins. E proteins promote T cell commitment and differentiation from DN1 to DN3 stages. Pre-TCR and TCR signaling in αβ T cells lead to transient Id3 up-regulation and E protein inhibition. In γδ T cell development, a gradient of γδ TCR signal determines the outcomes of different γδ subsets through regulation of Id3 expression and E protein activities. NKs, ILC2s and ILC precursors (ILCPs) may arise in the DN stages when E protein functions are suppressed. The figure was created by BioRender.com.

shown to be gate-keepers in the crossroads to T cell and ILC fates but the downstream transcriptional events remain to be further elucidated as the technologies and critical reagents become available.

Author contributions

AP and X-HS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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