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E and Id Proteins Influence Invariant NKT Cell Sublineage Differentiation and Proliferation

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Disease outcome is known to be influenced by defined subsets of invariant NKT (iNKT) cells residing in distinct locations within peripheral tissue. However, the factors governing the development of these unique iNKT sublineages during thymic development are unknown. In this study we explored the mechanism by which E protein transcription factors and their negative regulators, the Id proteins, control the development of iNKT sublineages after positive selection. We found that E proteins directly bound the promyelocytic leukemia zinc finger (PLZF) promoter and were required for expression of this lineage-defining transcription factor and for the maturation and expansion of thymic iNKT cells. Moreover, expression of the negative regulators of E proteins, Id2 and Id3, defined distinct iNKT cell sublineages. Id3 was expressed in PLZFhigh NKT2 cells and loss of Id3 allowed for increased thymic iNKT cell expansion and abundance of the PLZF+ NKT2 sublineage. Id2 was expressed in T-BET+ NKT1 cells, and both Id proteins were required for the formation of this sublineage. Thus, we provide insight into E and Id protein regulation of iNKT cell proliferation and differentiation to specific sublineages during development in the thymus. The Journal of Immunology, 2014, 192: 2227–2236.

N atural killer T cells are a unique subset of T cells able to recognize glycolipid Ags presented by the MHC class I–like molecule CD1d. The best studied NKT cell population utilizes an invariant TCR α-chain comprised of the V region 14 and the J region 18 (Vα14-Jα18) gene segments, and these cells are therefore termed invariant NKT (iNKT) cells. Within hours of activation, iNKT cells produce large amounts of numerous cytokines and thus play an important role in the early immune response to microbial pathogens. Additionally, iNKT cells are involved in protection from cancer and have been implicated in response to microbial pathogens. Furthermore, iNKT cells are involved in direct activation of promyelocytic leukemia zinc finger (PLZF) expression, the lineage-defining transcription factor of the NKT cell program, and the presence of PLZF allows iNKT cell differentiation from stage 2 to stage 3 in peripheral tissue, although a subfraction will mature and remain in the thymus (14). IL-15 and expression of the transcription factor T-BET are essential for this transition from stage 2 to stage 3, which is characterized by upregulation of NK1.1 (15, 16).

This concept of sequential, well-defined developmental stages of iNKT cells has recently been modified in the context of new findings. It is now appreciated that within the CD44+NK1.1+ stage 2 population there exists three subsets of iNKT cells: 1) cells that continue to differentiate, upregulating T-BET while downregulating PLZF, and produce IFN-γ upon stimulation (NKT1 cells); 2) cells that retain PLZF expression and produce IL-4 and IL-13 (NKT2 cells); and 3) cells that upregulate expression of retinoic acid–related orphan receptor (ROR)γt, while remaining low for PLZF and T-BET, and produce IL-17 (NKT17 cells) (1, 17, 18). Thus, it is likely that alterations in iNKT cell maturation that affect the transition from stage 2 to stage 3 will also affect differentiation of all three sublineages of iNKT cells. Currently, many of the factors that regulate the development of these individual subpopulations remain unknown.

E proteins are basic helix-loop-helix transcription factors. In lymphocytes, E47 and E12 (Tcf3), HEB (Tcf12), and E2-2 (Tcf4) bind to canonical E-box binding sites as homo- or heterodimers. In contrast, inhibitor of DNA binding (Id) proteins are able to heterodimerize with E proteins but, because they lack a DNA binding domain, act as dominant-negative regulators of E protein transcriptional activity (19). Of the four Id proteins, Id2...
and Id3 are most highly expressed in lymphocytes. E and Id proteins influence T cell development and formation of immunological memory (20–23). Furthermore, Id2 is required for survival of iNKT cells in the liver and bone marrow, whereas absence of Id3 leads to abundance of innate-like γδ T cells (24–27). Additionally, we previously found that the E protein transcription factor HEB allows for survival of CD4⁺CD8⁺ double-positive (DP) thymocytes. HEB promoted ROSyt and Bcl-X₁ expression, allowing secondary TCR rearrangements, including the canonical iNKT cell V₄-14-J₈ TCR gene rearrangement to occur (28). One finding of note in our previous study was that rescued of HEB-deficient mice with a V₄-14-J₈ transgene did not lead to complete recovery of iNKT cells, particularly in terms of their maturation state. Moreover, we observed high mRNA expression levels of both E2A and HEB at stage 0 during iNKT cell development. In the current study, we explore the mechanisms by which E and Id proteins control iNKT cell development and differentiation beyond TCR rearrangement.

Materials and Methods

**Mice**

Mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego. Tcf3²⁻/⁻ Tcf12⁻/⁻ Cd4cre⁻ and Tcf3²⁻/⁻ Tcf12⁻/⁻ Cd4cre⁺ mice were a gift from Y. Zhuang (Duke University), generated as described (22). V₄-14-J₈ transgenic mice were a gift from C. Murre and were generated as described (22). Id2-YFP mice were generated as previously described (29). V₄-14-J₈ TCR transgenic mice were a gift from A. Bendelac (University of Chicago) and M. Kronenberg (La Jolla Institute for Allergy and Immunology). Id3-GFP mice were a gift from C. Murre and were generated as described (22). Id2-YFP mice were generated as previously described (21).

Fetal liver chimeras were generated by transferring 5–10 x 10⁶ E14.5 fetal liver cells into lethally irradiated (1000 rad) CD45.1, recipient mice. Id2⁻/⁻ donor cells were CD45.2, WT donor cells were CD45.1, Id2⁻/⁻ donor cells were CD45.2, and recipient mice were CD45.1.2.

Mixed bone marrow chimeras were generated by transferring a mixture of 6 x 10⁶ B220⁺CD4⁻CD8⁻ bone marrow cells of a CD45.1 wild-type (WT) donor and 3 x 10⁶ B220⁺CD4⁺CD8⁻ bone marrow cells of CD45.2 Id2⁻/⁻ donor cells into lethally irradiated (1000 rad) CD45.1,2 recipient mice. All chimeras were rested for at least 8 wk to allow reconstitution of the host.

**Flow cytometry**

Single-cell suspensions were prepared from indicated tissues. Hepatic NKT cells were isolated from liver as previously described (28) using 34% Percoll solution (Sigma-Aldrich). The following Abs were used: TCRβ-FITC (clone H57-597), CD4-FITC (L3T4), CD8-FITC (53-6.7) (BD Pharmingen), CD45.2-PE-Cy5 (104), CD45.1-PE-Cy5 (104), NK1.1-PerCP-Cy5.5 (104), CD44-PE (clone H12F1) (BD Pharmingen), CD44-PE (clone H12F1) (BD Pharmingen), CD3-PE-Cy7 (14507) (BioLegend), CD44-PE-Cy7 (224940) (BioLegend). Abs were used as indicated. All samples were collected on an LSR II, FACS Fortessa, or FACSAria (BD Biosciences) fitted with custom glass mirrors from Omega filters (510/21 with 502LP or 505LP for GFP, 530/30 with 525LP for YFP) and were analyzed with FlowJo software (Tree Star).

**Quantitative PCR and chromatin immunoprecipitation**

Cells were sorted as indicated. RNA was extracted with TRIzol reagent (Invitrogen), treated with genomic DNA wipeout reagent (Qiagen), and cDNA was generated using a CDNA kit (Qiagen). The abundance of mRNA was assessed by quantitative PCR with npspecific product detection (SYBR Green; Stratagene) using primers that amplify in a linear relationship with primers for “housekeeping” genes. Results were normalized to expression of hypoxanthine phosphoribosyltransferase transcripts. Chromatin immunoprecipitation assays were performed as described (28).

A polyclonal Ab specific for E2A (V-18X) or HEB (A-20X) (Santa Cruz Biotechnology) was used to precipitate E2A-DNA or HEB-DNA complexes, and rabbit IgG Ab (2729; Cell Signaling Technology) was used as a negative control. Immunocomplexes were bound to protein G-Sepharose beads (Cell Signaling Technology) and were washed four times. DNA was eluted and purified and was analyzed by quantitative PCR to detect putative E protein binding sites in the zbtb16 gene. Sequences used were: Zbtb16Tctcgcaccaaggcccattacag and 3′-GGAGAAGATGACACAAA.

### Statistical analysis

Differences between data sets were analyzed by an unpaired two-tailed Student t test, Mann–Whitney U test, one-way ANOVA, or Bonferroni post hoc test where applicable.

### Results

**E proteins are required for iNKT cell development**

Although we previously detected high expression levels of E2A and HEB mRNA at stage 0 of iNKT cell development, indicating a possible requirement for E proteins during iNKT cell thymic development, we showed that loss of E protein expression led to impaired rearrangement of the canonical V₄-14-J₈ iNKT cell TCR (28). In the present study, we crossed V₄-14-J₈ transgenic (V₄-14tg) mice to mice conditionally deficient for Tcf3 (E2A) and Tcf12 (HEB) at the DP stage of thymocyte development (Tcf3²⁻/⁻ Tcf12⁻/⁻ Cd4cre⁻), thus bypassing the requirement for E proteins in TCR rearrangement. Both the frequency (Fig. 1A, 1B) and absolute number (Fig. 1C) of iNKT cells in the thymus, spleen, and liver were reduced in the E protein–deficient mice relative to WT mice, showing impaired iNKT cell development in the absence of E proteins.

Closer analysis revealed that E protein–deficient iNKT cell development was blocked at stage 0 (CD1d-tet⁺CD24⁺) and stage 1 (CD1d-tet⁺CD24⁻CD44⁺ NK1.1⁺) (Fig. 1D). This defect at stage 1 was also observed in iNKT cells in peripheral tissue (Fig. 1D). Interestingly, examination of absolute cell numbers in the thymus showed equal numbers of stage 0 and stage 1 iNKT cells in the absence of E proteins whereas there was a significant loss of cells at stages 2 and 3 (Fig. 1E). These data indicated a failure of thymic iNKT cells to progress through development in the absence of E2A and HEB and suggested a role for these proteins during iNKT cell differentiation, after positive selection to the lineage.

**E proteins regulate proliferation and PLZF expression during development of iNKT cells**

Given that iNKT cells failed to progress to stages 2 and 3 during thymic development, we next asked how E proteins might influence this process. By staining for Ki-67, a marker of actively dividing cells, we observed that the number of proliferating E protein–deficient cells at stage 1 was reduced by 50% compared WT cells (Fig. 2A). E protein–deficient cells at stage 0 also showed a slight increase in Ki-67 expression (Fig. 2A). Thus, failure of iNKT cells to accumulate at later stages of development in the absence of E proteins was attributable in part to the inability of iNKT cells to undergo the proliferative burst typically observed in these cells at stage 1. Tcf3⁻/⁻ Tcf12⁻/⁻ Cd4cre⁻ iNKT cells did not show an increase in active caspase-3⁺ and caspase-7⁺ iNKT cells at stages 0 and 1 relative to WT cells (data not shown), indicating that E proteins influence iNKT cell proliferation rather than survival at stage 1.

We were next interested in identifying the targets of E protein transcription factors during iNKT cell differentiation. One of the most prominent transcription factors, often referred to as the...
“master regulator” of iNKT cell development, is the protein PLZF encoded by the gene Zbtb16. Examining mRNA expression, we observed a 90% reduction of Zbtb16 expression in sorted iNKT cells from E protein−deficient animals relative to their WT counterparts at stages 0 and 1 when Zbtb16 is most highly expressed (Fig. 2B). Moreover, at the protein level, PLZF expression was also diminished by 84% in Tcf3^{f/f} Tcf12^{f/f}Cd4cre + iNKT cells at stage 1 (Fig. 2B). Examination of the Zbtb16 promoter revealed two conserved E-box sites, one in the 5' upstream region (E-box site 1) and one in the first intron upstream of the ATG start site (E-box site 2) (Fig. 2C). By using ChIP, we showed binding of E2A and HEB at both of these E-box sites in the Zbtb16 promoter. With sorted Tcf3^{f/f} Tcf12^{f/f}Cd4cre + iNKT cells at stage 0 as a negative control, we showed that E protein binding was specific at these two E-box sites (Fig. 2C). Although PLZF is required for iNKT cell development, notably it does not influence the proliferative burst observed as iNKT cells proceed through development (10). Thus, we conclude that E protein transcription factors dually influence iNKT cell development by regulating expression of the lineage-defining transcription factor PLZF and by regulating proliferation.

Id2 and Id3 expression define developmental stages and sublineages in thymic iNKT cells

E proteins are negatively regulated by the Id proteins, of which Id2 and Id3 are the Id family members most highly expressed in thymocytes. We previously showed the transcriptional regulator Id2 is essential for peripheral iNKT cell survival in hepatic tissue (25). In the present study, we wanted to explore the contribution of both Id2- and Id3-mediated control of E proteins to iNKT cell thymic development. By examining mRNA expression in sorted iNKT cells from different stages of thymic development, we noted that Id3 was most highly expressed at the early stages (stages 1 and 2) whereas Id2 was expressed more prominently in stage 3.
iNKT cells (Fig. 3A). By using reporter mice in which YFP or GFP was knocked into the first exon of the Id2 or Id3 gene, respectively, we similarly observed Id3-GFP expression is highest at the earliest stages of iNKT cell development, whereas Id2-YFP expression was most readily detected as the iNKT cells progressed through maturation stages in the thymus (Fig. 3B).

By gating on the recently described iNKT cell sublineages, defined by expression of unique transcription factors in the thymus (1, 17, 18), we observed that each sublineage had a distinct Id protein expression profile: Id2-YFP expression was highest in T-BET⁺ NKT1 cells, whereas Id3-GFP remained high in PLZF high NKT2 cells. RORγt⁺ NKT17 cells were intermediate for both Id2 and Id3 (Fig. 3B, 3C). Finally, by gating on NK1.1² cells, we could identify a population of T-BET⁺ cells potentially representing a population of immature NKT1 cells. Comparing NK1.1⁻ T-BET⁺ to NK1.1⁺ T-BET⁺ cells in the thymus, we noted that Id3 expression was significantly higher in the NK1.1⁺ T-BET⁺ immature iNKT cells (Fig. 3D). We speculate that as NKT1 cells mature and upregulate NK1.1 expression, Id3 expression is downregulated whereas Id2 expression continues to be upregulated. We found that Id2 and Id3 mark these different NKT sublineages, implying distinct regulation and/or function of these Id protein family members.

Loss of Id2 and Id3 affect iNKT cell abundance and development

To examine the roles of Id2 and Id3 in thymic iNKT cell development, we used mice homozygous for the knock-in sequence

FIGURE 2. E proteins regulate proliferation and PLZF expression in thymic iNKT cells. (A) Ki-67 expression by stage 0 and stage 1 CD1d-tet⁺ TCRb⁺ gated iNKT thymocytes from the indicated genotypes. Numbers indicate percentage Ki-67⁺ cells within the gate. Bar graphs indicate average frequency (±SEM) of CD1d-tet⁺ TCRb⁺ gated iNKT Ki-67⁺ cells at stages 0 and 1. Graphs are an average of six mice from three individual experiments. Statistical significance was determined using an unpaired two-tailed t test. *p < 0.05. (B) Bar graphs indicate relative expression of zbtb16 mRNA by stage 0 and 1 CD1d-tet⁺ TCRb⁺ gated iNKT cells determined by quantitative PCR. Data are normalized to Vα14-Jα18tg⁺ Tcf3⁺ Tcf12⁺ Cd4cre⁻ cells and are the average of three individual samples from three independent experiments. Histogram plots indicate PLZF expression by stage 0 and 1 CD1d-tet⁺ TCRb⁺ gated iNKT cells from the indicated genotypes. Numbers indicate percentage of PLZF high cells within the gate. Data are representative of three independent experiments with n = 1–3 mice/group. Statistical significance was determined using an unpaired two-tailed t test. ***p < 0.0005, ****p < 0.0001. (C) The zbtb16 promoter is a direct target of E2A and HEB. Schematic indicates position of E-box sites in the zbtb16 promoter relative to the ATG start site. Thymic iNKT cells from the indicated genotypes were sorted at stage 0 (CD1d-tet⁺ TCRb⁺CD24⁺), and chromatin was precipitated with anti-E2A, anti-HEB, or control IgG Abs. The relative amounts of input or precipitated DNA containing the indicated E-box sites were determined by quantitative PCR. Fold change of Vα14-Jα18tg⁺ Tcf3⁺ Tcf12⁺ Cd4cre⁻ pull-down over Vα14-Jα18tg⁺ Tcf3⁻ Tcf12⁻ Cd4cre⁻ control are graphed and are an average of sorted cells from three mice for Vα14-Jα18tg⁺ Tcf3⁺ Tcf12⁻ Cd4cre⁻ and two mice for Vα14-Jα18tg⁺ Tcf3⁻ Tcf12⁻ Cd4cre⁻.
encoding *YFP* into *Id2* (*Id2*Y/Y) or *GFP* into *Id3* (*Id3*G/G), thus generating *Id2*- or *Id3*-deficient mice. Mice deficient for *Id2* die at approximately 2 wk of age on the B6 background; therefore, we generated fetal liver chimeras (FLC), transferring congenically marked E14.5 fetal liver cells into irradiated hosts. As we have previously shown that *Id3* expression increased in CD8+ effector T cells as well as innate-like and effector T cells in the absence of *Id2* (21). Thus, we were interested in addressing the possible compensatory roles for *Id2* and *Id3* during iNKT cell development. Examining *Id3*-GFP expression in *Id2*-deficient iNKT cells, we noted that *Id3* expression was dramatically increased in these cells in the thymus, spleen, and liver (Fig. 4C). Moreover, *Id2*-YFP expression decreased slightly with loss of *Id3* (*Id3*G/G) (Fig. 4C). Thus, as in the case of CD8+ effector T cells, it appears *Id2* can negatively regulate *Id3* expression whereas *Id3* may positively regulate *Id2* expression (21).

**Id3 limits abundance of iNKT cells**

*Id3* germline deficiency results in a complex T and B cell phenotype with an increase in γδ T cells as well as innate-like and effector memory–like T cells (22, 24, 26, 27). To assess the cell-intrinsic role of *Id3* in iNKT cell development without possible influence of these developmental defects, we generated mixed bone marrow chimeras using equal numbers of *Id3*-sufficient (*Id3*G/G) and *Id3*-deficient (*Id3*G/G) bone marrow cells to reconstitute irradiated host...
mice. Loss of Id3 in these mixed bone marrow chimeras led to a sharp increase in both the frequency and number of iNKT cells in the thymus, spleen, and liver whereas limited numbers of WT iNKT cells were recovered, making comparative analysis of iNKT cell subsets difficult (data not shown). It is also possible that in this system increased numbers of γδ T cells might influence the development of both the WT and Id3-deficient iNKT cells. Thus, to obtain sufficient numbers of WT iNKT cells, we next mixed Id3 WT and Id3-deficient bone marrow cells at a 2:1 ratio. We again observed increased frequency and absolute number of the Id3-deficient iNKT cells (Fig. 5A, 5B), whereas conventional T cell numbers reflected the 2:1 ratio of the engrafted bone marrow cells in these mice (Fig. 5B). The difference in iNKT cell numbers between the germline knockout FLC and the Id3+/+ Id3G/G mixed bone marrow chimeras may be accounted for by the presence of increased innate-like γδ T cell numbers in the germline knockout FLC, as these cells compete with iNKT cells for space within the thymic niche (30, 31). Further analysis of iNKT cell differentiation indicated that stage 0 cells were unaffected by Id3 deficiency in the mixed chimeras (Fig. 5C, 5D). However, the Id3-deficient iNKT cells recovered from the mixed bone marrow chimeras were present in increased absolute numbers at stages 2 and 3, as they developed in the thymus (Fig. 5D). Thus, we conclude that negative regulation of E proteins by Id3 is an essential process that must occur as iNKT cells progress through thymic differentiation and that loss of Id3 and continued E protein activity lead to abundance of iNKT cells.

**FIGURE 4.** Compensation by Id2 and Id3 as iNKT cells differentiate in the thymus. Flow cytometric analysis showing CD44+NK1.1+ expression by CD1d-tet+TCRβ+ gated iNKT cells from (A) WT, Id2-deficient (Id2Y/Y), and Id2-deficient Id3-heterozygote (Id2Y/YId3G/G) FLC and (B) WT, Id3-deficient (Id3G/G), and Id3-deficient Id2-heterozygote (Id3G/GId2Y/Y) FLC. Bar graphs indicate iNKT cell frequency and absolute number at stages 1, 2, and 3 of the indicated genotype. (C) Histograms and bar graphs indicate Id3-GFP expression by Id2-deficient cells and Id2-YFP expression by Id3-deficient cells for CD1d-tet+TCRβ+ gated iNKT cells in the thymus, spleen, and liver. Data shown are an average (±SEM) of four fetal liver chimeras per group, representative of two independent experiments. Statistical significance was determined using a one-way ANOVA or unpaired two-tailed t-test. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.

**Id2 and Id3 regulate iNKT cell proliferation and sublineage commitment**

Because we observed higher iNKT cell numbers during thymic development in the absence of Id3 (Fig. 5D), we examined proliferation and cell death of Id3-deficient iNKT cells in the mixed bone marrow chimeras. An abundance of Ki-67+ iNKT cells could be readily detected among Id3-deficient iNKT cells relative to WT cells in the thymus of mixed bone marrow chimeras, whereas proliferation remained unchanged in peripheral organs (Fig. 6A and data not shown). By observing different stages of iNKT cell development in the thymus, we found that more Id3G/G cells were actively dividing than their WT counterparts at stages 0–1 and, in particular, at stage 2 (Fig. 6B). Interestingly, in Id2 and Id3 reporter mice, proliferating iNKT cells in the thymus expressed more Id3 but less Id2 than did nonproliferating NKT cells (data not shown). Thus loss of Id3, which should result in enhanced E protein activity, led to increased and prolonged proliferation at the early stages of iNKT cell development; conversely, loss of E protein activity abrogated proliferation at this crucial stage of development (Figs. 2A, 6B).

We next examined the iNKT cell sublineages in the Id3+/+Id3G/G mixed bone marrow chimeras. More iNKT cells expressed PLZF in the absence of Id3, in particular iNKT cells at stage 2, reinforcing our data indicating that PLZF expression is positively regulated by E proteins (Figs. 2B, 2C, 6B). In contrast, fewer Id3-deficient iNKT cells at stage 2 expressed T-BET, consistent with the reduced frequency of Id3G/G iNKT cells reaching stage 3.
By examining PLZF and T-BET expression together, we clearly observed more Id3-deficient iNKT cells expressing PLZF alone (Fig. 6C). These data suggest E proteins or E protein–driven PLZF expression may negatively impact T-BET expression during iNKT cell development. RORγt+ cells were infrequent in both WT and Id3-deficient iNKT cells but trended toward a slightly higher frequency in the Id3-deficient population. Consequently, we examined the frequency of T-BET+ NKT1, PLZFhigh NKT2, and RORγt+ NKT17 iNKT cells in the presence or absence of Id3. The relative frequency of the T-BET+ NKT1 population was decreased in the absence of Id3, whereas the PLZFhigh NKT2 population was increased in the thymus (Fig. 6D). Moreover, the absolute number of PLZFhigh NKT2 cells was increased in the absence of Id3 in the thymus (Fig. 6D). Thus, we conclude that Id2 marks and is necessary for the differentiation and maturation of the T-BET+ NKT1 cells in the thymus.

**Discussion**

In this study, we have shown that E protein transcription factors and Id proteins together act to orchestrate the differentiation, proliferation, and survival of iNKT cells during thymic development. E2A and HEB both bound the promoter of Zbtb16, initiating expression of the transcription factor PLZF during the earliest stages of iNKT cell development. Furthermore, E proteins promoted iNKT cell proliferation at this crucial developmental stage, and loss of E2A and HEB led to a paucity of mature iNKT cells in peripheral tissues. Id proteins, in turn, influenced subsequent stages of iNKT cell development. Id3 was required for regulating iNKT cell expansion and its loss led to a dramatic increase in iNKT cell numbers. Moreover, a sublineage of iNKT cells identified by constitutively high PLZF expression (PLZFhigh NKT2...
cells) could also be identified by high Id3 expression; Id3 deficiency increased the abundance of PLZF high NKT2 cells, indicating that sustained E protein DNA-binding activity positively regulates this sublineage. Finally, high Id2 expression denoted NK1.1+T-BET+ NKT1 cells and was essential for the survival of these cells in the periphery.

Both E and Id proteins influence the proliferative burst that occurs by newly committed iNKT cells after positive selection. We observed lower proliferative capacity in the absence of E proteins whereas an increase in proliferation was observed in the absence of Id3, particularly at stages 1 and 2. Our previous work indicated that the E protein HEB was necessary for expression of Bcl-xL at the DP stage of T cell development, and indeed cells were susceptible to death in the absence of HEB (28). In the present study we found that E proteins also influence proliferation at subsequent stages of iNKT cell development, resulting in a similar phenotype to that observed owing to deficiency of the transcription factor c-MYC (12, 13). It is possible that E proteins regulate c-MYC expression directly or that both proteins collaborate to promote the proliferative burst. Interestingly, such a synergy has recently been suggested to occur during the development of Burkitt lymphoma (33).

Recent studies have begun uncovering how expression of the iNKT cell lineage–defining transcription factor PLZF is regulated. The transcription factor EGR2 is capable of binding the Zbtb16 promoter and indeed regulates expression of PLZF (9). We did not find evidence for regulation of EGR2 expression by E proteins, and thus we favor the idea that EGR2 and E proteins independently regulate PLZF expression during iNKT cell development. Unlike E protein–deficient iNKT cells that do not express PLZF, iNKT cells expressing Id3 are positive for PLZF. This suggests that Id3 reduces but does not abolish E protein transcriptional activity completely.

**Figure 6.** Id3 regulates proliferation and sublineage commitment in iNKT cells. Mixed bone marrow chimeras were generated by transfer of a 2:1 mix of Id3<sup>+/+</sup> to Id3<sup>G/G</sup> bone marrow to irradiated recipients. (A) Histograms showing Ki-67 staining by gated WT (Id3<sup>+/+</sup>) (black line) and Id3-deficient (Id3<sup>G/G</sup>) (green line) iNKT cells from the thymus. Bar graphs indicate average frequency (±SEM) of Ki-67<sup>+</sup> iNKT cells. (B) Histograms showing Ki-67, PLZF, and T-BET expression by CD1d-tet<sup>+</sup>TCRβ<sup>+</sup> gated WT (Id3<sup>+/+</sup>) (black line) and Id3-deficient (Id3<sup>G/G</sup>) (green line) iNKT cells at stage 0–1, stage 2, and stage 3 in the thymus. Bar graphs indicate frequency of Ki-67<sup>+</sup>, PLZF<sup>hi</sup>, and T-BET<sup>+</sup> cells by CD1d-tet<sup>+</sup>TCRβ<sup>+</sup> gated Id3<sup>+/+</sup> and Id3<sup>G/G</sup> iNKT thymocytes at indicated developmental stages. Data are average (±SEM) of three mice, representative of three independent experiments. (C) Flow cytometry plot indicating PLZF and T-BET expression by WT (Id3<sup>+/+</sup>) (black) and Id3-deficient (Id3<sup>G/G</sup>) (green) iNKT thymocytes. (D) Graphs of relative frequency and absolute number of T-BET<sup>+</sup> NKT1, PLZF<sup>hi</sup> NKT2, and ROR<sup>γt</sup> NKT17 cells of total gated Id3<sup>+/+</sup> and Id3<sup>G/G</sup> iNKT thymocytes. Data are average of five mice, representative of three independent experiments. (E) Bar graphs indicate frequency of Ki-67<sup>+</sup>, PLZF<sup>+</sup>, and T-BET<sup>+</sup> cells by CD1d-tet<sup>+</sup>TCRβ<sup>+</sup> gated Id2<sup>+/+</sup> and Id2<sup>Y/Y</sup> iNKT thymocytes at indicated developmental stages. Data are averages (±SEM) of six mice, representative of two independent experiments. (F) Graphs of relative frequency and absolute number of T-BET<sup>+</sup> NKT1, PLZF<sup>hi</sup> NKT2, and ROR<sup>γt</sup> NKT17 cells of total gated Id2<sup>+/+</sup> and Id2<sup>Y/Y</sup> iNKT thymocytes. Data are averages of six mice (Id2<sup>+/+</sup>) and three mice (Id2<sup>Y/Y</sup>), representative of three independent experiments. Statistical significance was determined using a Mann–Whitney U test and an unpaired two-tailed t test where applicable. *p < 0.05, **p < 0.005, ***p < 0.0005.
The regulation of Id2 expression in iNKT cells during development is still being elucidated. It has been reported that in myeloid cells PLZF can directly bind the Id2 promoter, whereas in iNKT cells EGR2 expression was essential for the upregulation of Id2 (9, 35). Furthermore, we have previously shown that active STAT4 and STAT5 directly bind the Id2 promoter to increase its expression in T cells and dendritic cells (21, 36). Interestingly, EGR2 directly induces transcription of Id2rb in iNKT cells, and both IL-2RB (CD122) and its ligand IL-15 are essential for the formation of stage 3 and presumably NKT1 cells (9, 37). We therefore hypothesize that EGR2 enables IL-15 signaling, which in turn activates STAT5 to induce Id2 expression. Thus, Id2 and Id3 differentially regulate E protein binding to DNA, potentially forming a gradient of transcriptional activity that influences iNKT cell development and proliferation.

A paradigm shift has recently been appreciated in the differentiation program that occurs as iNKT cells undergo development in the thymus. Terminally differentiated PLZFhigh NKT2 and RORγt+ NKT17 cells were found within the stage 2 CD44+ NK1.1+ population (1, 17, 18, 38). We found that whereas Id2 is highly expressed by T-BET+ NKT1 cells, Id3 is expressed by PLZFhigh NKT2 cells. We propose, as recently suggested by Hu et al. (39), that Id3 may be a unique transcriptional regulator associated with the NKT2 sublineage. Although the cytokine milieu required for the maintenance of Id3+ NKT2 cells has not been clearly identified, our previous analysis in CD8+ effector cells showed that Id3 expression was downregulated in the presence of IL-2, IL-12, and IL-21 (21). It is possible that Id3 expression is maintained by IL-25, as NKT2 cells reportedly express high levels of IL-17RB (5, 39, 40). Indeed, preliminary data indicate that Id3–GFP+ iNKT cells have higher expression levels of IL-17RB than do Id3–GFP– iNKT cells (A.W. Goldrath, unpublished observations and Ref. 39). Our previous data further support the notion that Id2+ NKT1 cells may be sensitive to IL-12 signaling, as these cells constitutively express IL-12R (1, 17), CD8+ effector T cells upregulate Id2 expression upon stimulation with IL-12, and STAT4 can bind and promote Id2 expression (21). Indeed, recent data by Watarai et al. (5) showed that Stat4 mRNA levels were upregulated in NKT1 cells relative to NKT2 cells. Thus, we postulate that in the periphery the cytokine milieu within a particular organ in which the iNKT cell sublineage resides will influence the homeostasis and survival of both Id2+ NKT1 and Id3+ NKT2 cells. Interestingly, RORγt+ NKT17 cells displayed comparatively low expression of both Id2 and Id3, implying increased E protein activity in this sublineage. This observation is consistent with our previous finding that HEB can directly bind to the Rorc promoter to induce RORγt expression (28). However, with loss of Id3 and increased E protein transcriptional activity, we found only a slight increase in the frequency of the RORγt+ NKT subset, suggesting that E protein activity alone is not sufficient for commitment to this sublineage. Other transcription factors are likely to block RORγt+ NKT commitment in a dominant fashion. Indeed, the transcription factor Th-Pok has recently been described to inhibit RORγt expression and NKT17 sublineage commitment (41).

We consistently found that loss of three Id protein alleles led to a decrease in both the frequency and absolute number of mature CD44+NK1.1+ iNKT cells, whereas loss of Id3 alone affected immature iNKT cell populations. Loss of both Id2 alleles affected mature CD44+NK1.1+ iNKT cells, a defect exacerbated by loss of one allele of Id3. Thus, although Id2 and Id3 can compensate for one another to some degree during early iNKT cell development, Id2 is required for formation of mature CD44+NK1.1+ iNKT cells whereas Id3 is necessary for formation of NKT2 cells, roles that cannot be compensated for by unhindered expression of Id3 or Id2, respectively. These data are consistent with two recent publications. Continuous E protein activity, using a conditional mouse model, led to an increase in NKT2 and NKT17 cells with a corresponding loss of NKT1 cells (39). Similarly, loss of both Id2 and Id3, using a conditional knockout approach, led to increased iNKT cell number (31). Thus, it appears that Id2 and Id3 play unique noncompensatory roles in the formation of distinct sublineages of iNKT cells.

With loss of Id3 and increased E protein activity, we found a considerable expansion of the PLZFhigh NKT2 subset at the expense of the T-BET+ NKT1 subset. Such changes in iNKT cell polarization imply higher susceptibility to development of colitis or asthma while maintaining increased protection from Th2-driven infections. NKT2 cells have been reported to play a role in the induction of airway hyperresponsiveness (5, 40, 42). With abundant PLZFhigh NKT2 cells, we would predict an increased susceptibility to airway hyperresponsiveness. Similarly, a greater frequency of PLZFhigh NKT2 cells was identified in the mesenteric lymph nodes, particularly in BALB/c mice (5). NKT cells have been associated with increased pathology in mouse models of ulcerative colitis, and these cells reportedly produce high levels of IL-4 and IL-13, suggesting that the iNKT cells residing in the large intestine may be part of the PLZFhigh NKT2 sublineage (43, 44).

E protein transcription factors and their negative regulators the Id proteins have many important functions during lymphocyte development. We show in this study that E proteins control proliferation after positive selection and regulation of PLZF expression. Id2 and Id3 define and influence commitment to the T-BET+ NKT1 and PLZFhigh NKT2 populations, respectively. Our findings indicate that E proteins and Id proteins dictate the development, differentiation, and eventual iNKT cell polarization and abundance observed in health and disease.

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References


