



Published in final edited form as:

J Immunol. 2014 March 1; 192(5): 2227–2236. doi:10.4049/jimmunol.1302904.

E and Id proteins influence invariant Natural Killer T cell sublineage differentiation and proliferation

Louise M. D'Cruz^{1,*}, Martin H. Stradner^{1,2,*}, Cliff Y. Yang¹, and Ananda W. Goldrath¹

¹University of California San Diego, Division of Biology, 9500 Gilman Drive, La Jolla, CA 92093-0377

²Medical University of Graz, Division of Rheumatology and Immunology, Auenbruggerplatz 15, Graz A-8035, Austria

Abstract

Disease outcome is known to be influenced by defined subsets of invariant Natural Killer T (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. Here 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 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 PLZF^{high} NKT2 cells and loss of Id3 allowed for increased thymic iNKT cell expansion and abundance of the PLZF⁺ NKT2 sublineage. Id2 was expressed in TBET⁺ 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.

Introduction

Natural Killer T (NKT) cells are a unique subset of T cells able to recognize glycolipid antigens presented by the MHC class I-like molecule CD1d. The best-studied NKT cell population utilizes an invariant T cell receptor (TCR) α -chain comprised of the variable region 14 and the joining region 18 (V α 14-J α 18) 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. In addition, iNKT cells are involved in protection from cancer and have been implicated in autoimmune diseases such as ulcerative colitis and type 1 diabetes (1-3). As iNKT cell number and function are associated with these diseases and vary broadly in humans and different mouse strains (4, 5), it is essential to understand the mechanisms driving iNKT cell maturation and differentiation.

iNKT cells undergo positive selection, expansion and early maturation in the thymus where four developmental stages have been defined based on the expression of CD24, CD44 and

Address all correspondence and reprint requests to Ananda Goldrath, University of California, San Diego, Division of Biological Sciences, 9500 Gilman Drive, La Jolla, CA 92093-0377. Telephone number: 858-534-5839. agoldrath@ucsd.edu.

*These authors contributed equally to this work

FOOTNOTES: This work was supported by PEW Scholars (A.W.G.), Leukemia and Lymphoma Society Scholar (A.W.G) and Fellow (L.M.D) awards and grants from the NIH (AI067545 and AI072117) to A.W.G.; KAI097286A to L.M.D. and the Austrian Science Fund (FWF): [J 3189-B11] to M.H.S.

NK1.1; this understanding of iNKT cell development is used by many studies (2, 6, 7). Upon rearrangement of the canonical V α 14-J α 18 TCR and positive selection by CD1d-expressing cortical thymocytes, commitment to the iNKT cell lineage is observed by cells expressing CD24 (stage 0) (2, 6, 7). Subsequently, iNKT cells downregulate CD24 expression transitioning to the highly proliferative CD24⁻CD44⁺NK1.1⁻ stage 1, a process dependent on both EGR2 and NF- κ B transcription factors (6, 8, 9). EGR2 is involved in direct activation of PLZF expression, the lineage-defining transcription factor of the NKT cell program, and the presence of PLZF allows iNKT cell progression from stage 1 to CD44⁺NK1.1⁻ stage 2 (9-11). At stages 1 and 2, iNKT cells undergo extensive proliferation, which is abrogated in the absence of the transcription factor c-MYC (12, 13). Subsequently, many stage 2 iNKT cells exit the thymus to complete maturation 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 TBET 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 TBET 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 ROR γ t, while remaining low for PLZF and TBET, 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, as 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 $\gamma\delta$ T cells (24-27). In addition, we previously found that the E protein transcription factor HEB allows for survival of CD4⁺CD8⁺ double positive (DP) thymocytes. HEB promoted ROR γ t and BCL-XL expression, allowing secondary TCR α rearrangements including the canonical iNKT cell V α 14-J α 18 TCR α gene rearrangement to occur (28). One finding of note in our previous study was that rescue of HEB-deficient mice with a V α 14-J α 18 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 α 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. *Tcf3^{fl/fl}Tcf12^{fl/fl}Cd4cre⁻* and *Tcf3^{fl/fl}Tcf12^{fl/fl}Cd4cre⁺* mice were a gift from Y. Zhuang (Duke University), generated as previously described (29). V α 14-J α 18 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 × 10⁶ E14.5 fetal liver cells into lethally irradiated (1000 rad) CD45.1.2 recipient mice. *Id2*^{Y/Y} donor cells were CD45.2, WT donor cells were CD45.1, *Id3*^{G/G} donor cells were CD45.2, and recipient mice were CD45.1.2.

Mixed bone marrow chimeras were generated by transferring a mixture of 6 × 10⁶ B220⁻CD4⁻CD8⁻ bone marrow cells of a CD45.1 wild-type donor and 3 × 10⁶ B220⁻CD4⁻CD8⁻ bone marrow cells of CD45.2 *Id3*^{G/G} donor into lethally irradiated (1000 rad) CD45.1.2 recipient mice. All chimeras were rested for at least eight weeks 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). The following antibodies were used: TCRβ FITC (clone H57-597), CD4 FITC (L3T4), CD8 FITC (53-6.7) (Biolegend), CD24 FITC (M1/69) (BD Pharmingen), CD45.1 FITC (A20), CD45.2 FITC (104), CD1d Tetramer Alexa 488 (NIH Tetramer Core Facility (mCD1d/PBS57)), CD1d Tetramer PE (NIH Tetramer Core Facility (mCD1d/PBS57)), TCRβ PE (H57-597), PLZF PE (Mags.21F7), NK1.1 PerCP Cy5.5 (PK136), CD45.2 PerCP Cy5.5 (104), NK1.1 APC (PK136), TCRβ APC (H57-597), CD45.2 APC (104), CD44 APC (IM7), CD4 APC (L3T4) (BD Pharmingen), CD8 APC (53-6.7) (Biolegend), TBET APC (4B10), CD1d Tetramer Alexa 647 (NIH Tetramer Core Facility (mCD1d/PBS57)), CD19 PeCy7 (ID3), CD45.1 PeCy7 (A20), CD45.2 Alexa 750 (104), Ki-67 Pacific Blue (SolA15). All antibodies were purchased from eBioscience unless otherwise specified. Samples were collected on a FACS LSRII, FACS Fortessa or FACS Aria (BD Biosciences) fitted with custom mirrors from Omega Filters (510/21 with 502LP or 505LP for GFP, 530/30 with 525LP for YFP) and were analyzed with FlowJo software (TreeStar).

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 cDNA kit (Qiagen). The abundance of mRNA was assessed by quantitative PCR with nonspecific product detection (SYBR Green; Stratagene) using primers that amplify in a linear relationship with primers for 'housekeeping' genes. Results were normalized to expression of HPRT transcripts. Chromatin-immunoprecipitation assays were performed as described (28). A polyclonal antibody specific for E2A (V-18X) or HEB (A-20X) (Santa Cruz Biotechnology) was used to precipitate E2A-DNA or HEB-DNA complexes, and rabbit immunoglobulin G antibody (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. *Zbtb16* ChIP primer sequences E box site 1: 5' gggttctctggttgctgct and 3' agccttgctgtacaaaga. *Zbtb16* ChIP primer sequences E box site 2: 5' caccggaatgcacaggag and 3' gggagaaaaggatgcacaaa.

Statistical Analysis

Differences between data sets were analyzed by an unpaired two-tailed student's 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

While 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 loss of E protein expression led to impaired rearrangement of the canonical V α 14-J α 18 iNKT cell TCR (28). Here we crossed V α 14-J α 18 transgenic (V α 14^{tg}) mice to mice conditionally deficient for *Tcf3* (E2A) and *Tcf12* (HEB) at the DP stage of thymocyte development (*Tcf3^{fl/fl}Tcf12^{fl/fl}Cd4cre⁺*), thus bypassing the requirement for E proteins in TCR rearrangement. Both the frequency (Fig. 1A and 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 wild type mice, showing impaired iNKT cell development in the absence of E proteins.

Closer analysis revealed 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 while 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. 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 wild type 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^{fl/fl}Tcf12^{fl/fl}Cd4cre⁺* iNKT cells did not show an increase in active caspase 3 and 7 positive iNKT cells at stage 0 and 1 relative to wild type cells (data not shown), indicating 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 wild type 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^{fl/fl}Tcf12^{fl/fl}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). Using chromatin immunoprecipitation (ChIP), we showed binding of E2A and HEB at both of these E box sites in the *Zbtb16* promoter. With sorted *Tcf3^{fl/fl}Tcf12^{fl/fl}Cd4cre⁺* iNKT cells at stage 0 as a negative control, we showed 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 (Kovalovsky et al., 2008). Thus, we conclude 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). Here we wanted to explore the contribution of both Id2- and Id3-mediated control of E proteins to iNKT cell thymic development. Examining mRNA expression in sorted iNKT cells from different stages of thymic development, we noted Id3 was most highly expressed at the early stages (stages 1 and 2) while Id2 was expressed more prominently in stage 3 iNKT cells (Fig. 3A). 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, while Id2-YFP expression was most readily detected as the iNKT cells progressed through maturation stages in the thymus (Fig. 3B).

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 TBET⁺ NKT1 cells, while Id3-GFP remained high in PLZF^{high} NKT2 cells. RORγt⁺ NKT17 cells were intermediate for both Id2 and Id3 (Fig. 3B and 3C). Finally, gating on NK1.1⁻ cells, we could identify a population of TBET⁺ cells, potentially representing a population of immature NKT1 cells. Comparing NK1.1⁻TBET⁺ to NK1.1⁺TBET⁺ cells in the thymus, we noted Id3 expression was significantly higher in the NK1.1⁻TBET⁺ immature iNKT cells (Fig. 3D). We speculate that as NKT1 cells mature and upregulate NK1.1 expression, Id3 expression is downregulated while 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 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 two weeks 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, using Id2-germline knock-out FLC (*Id2^{Y/Y}*), thymic development of iNKT cells in the absence of Id2 is relatively normal and Id2 expression is essential for the survival of peripheral iNKT cells (25). Interestingly, loss of Id2 minimally affected thymic development of iNKT cells, most likely due to a role of Id3 during the early stages of iNKT cell development (Fig. 4A). In Id3-deficient FLC (*Id3^{G/G}*), we similarly observed no significant difference in the frequency or cell number of iNKT cells in the thymus, however Id3-deficiency led to a decrease in the absolute number of iNKT cells in peripheral tissues (data not shown).

We next asked how iNKT cell development would be affected by loss of both *Id2* and *Id3*. However, our efforts to generate *Id2-Id3*-double deficient FLC were unsuccessful, most likely due to requirement for these proteins during embryonic development. Instead, we continued our analysis of compound homo- and heterozygote Id2- and Id3-deficient mice and examined iNKT cell thymic development in Id2-deficient Id3-heterozygote (*Id2^{Y/Y}Id3^{G/+}*) FLC relative to wild-type (WT) and Id2-deficient (*Id2^{Y/Y}*) FLC (Fig. 4A). Here we observed a 5-fold increase in frequency and 2-fold increase in absolute number of iNKT cells at stage 1 relative to wild-type cells and a significant decrease ($P < 0.0005$) in

iNKT cells at stage 3 (Fig. 4A). This phenotype was more pronounced in *Id3*-deficient *Id2*-heterozygote (*Id3^{G/G}Id2^{Y/+}*) iNKT cells (Fig. 4B). *Id3^{G/G}Id2^{Y/+}* iNKT cells were significantly increased in frequency and absolute number ($P < 0.005$) at stage 1 and were decreased more than 60% at stage 3 relative to wild-type cells (Fig. 4B). We postulate that in thymic iNKT cells, Id proteins are required to regulate iNKT cell abundance and allow for progression to stage 3 while loss of three Id protein alleles led to a decrease in CD44⁺ NK1.1⁺ mature iNKT cells (Fig. 4A and 4B).

We have previously shown that *Id3* expression increased in CD8⁺ 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 (*Id2^{Y/Y}*), 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 while *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 $\gamma\delta$ T cells as well as innate-like and effector-memory-like T cells (22, 24, 26, 27). In order 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^{+/+}*) 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 while 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 $\gamma\delta$ T cells might influence the development of both the WT and *Id3*-deficient iNKT cells. Thus, in order 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 and 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-knock-out FLC and the *Id3^{+/+}Id3^{G/G}* mixed bone marrow chimeras may be accounted for by the presence of increased innate-like $\gamma\delta$ T cell numbers in the germline knock out FLC, as these cells compete with iNKT cells for space within the thymic niche (30, 31). Further analysis of iNKT cell differentiation indicated stage 0 cells were unaffected by *Id3* deficiency in the mixed chimeras (Fig. 5C and 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 leads to abundance of iNKT cells.

Id2 and Id3 regulate iNKT cell proliferation and sublineage commitment

Since 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 wild type cells in the thymi of mixed bone marrow chimeras, while proliferation remained unchanged in peripheral organs (Fig. 6A and data not shown). Observing different stages of iNKT cell development in the thymus, we found that more *Id3^{G/G}* cells were actively dividing than their wild type

counterparts at stages 0-1 and stage 2, in particular (Fig. 6B). Interestingly, in *Id2* and *Id3* reporter mice, proliferating iNKT cells in the thymus expressed more *Id3* but less *Id2* than non-proliferating 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 (Fig. 6B and 2A).

We next examined the iNKT cell sublineages in the *Id3^{+/+}/Id3^{G/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 PLZF expression is positively regulated by E proteins (Fig. 6B, 2B and 2C). In contrast, fewer *Id3*-deficient iNKT cells at stage 2 expressed TBET, consistent with the reduced frequency of *Id3^{G/G}* iNKT cells reaching stage 3 (Fig. 5D and 6B). Examining PLZF and TBET 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 TBET expression during iNKT cell development. *ROR γ ⁺* cells were infrequent in both WT and *Id3*-deficient iNKT cells but trended towards a slightly higher frequency in the *Id3*-deficient population. Consequently, we examined the frequency of TBET⁺ NKT1, PLZF^{high} NKT2, and *ROR γ ⁺* NKT17 cells in the presence or absence of *Id3*. The relative frequency of the TBET⁺ NKT1 population was decreased in the absence of *Id3*, while the PLZF^{high} NKT2 population was increased in the thymus (Fig. 6D). Moreover, the absolute number of PLZF^{high} NKT2 cells was increased in the absence of *Id3* in the thymus (Fig. 6D). Thus, our analyses indicate *Id3*-deficiency results in a significant increase in PLZF^{high} NKT2 cells, most likely accounted for by uncontrolled E protein activity within this sublineage.

Finally, we examined the iNKT cell sublineages in either *Id2^{+/+}* or *Id2^{Y/Y}* bone marrow chimeras. As expected, we observed no significant difference in proliferation at any stage during iNKT cell development in the thymus (Fig. 6E). However, *Id2*-deficiency resulted in an increase in PLZF⁺ cells at stage 3 and a decrease in the frequency of TBET⁺ expressing cells prior to stage 3, suggesting that uncontrolled E protein activity positively regulates PLZF expression and might negatively regulate TBET expression (Fig. 6E). These data are supported by previously published data showing *Id2*-deficient CD8⁺ effector T cells fail to upregulate TBET expression during infection (32). Examining the overall frequency and absolute number of TBET⁺ NKT1, PLZF^{high} NKT2, and *ROR γ ⁺* NKT17 iNKT cells in the presence or absence of *Id2*, we consistently observed a decrease in the TBET⁺ NKT1 population in the absence of *Id2* (Fig. 6F). Thus, we conclude *Id2* marks and is necessary for the differentiation and maturation of the TBET⁺ NKT1 cells in the thymus.

Discussion

Here we have shown 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, PLZF^{high} 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⁺TBET⁺ 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 while conversely 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). Here we find E proteins also influence proliferation at subsequent stages of iNKT cell development, resulting in a similar phenotype to that observed due to deficiency in 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). Furthermore, c-MYC and E protein transcription factors are able to directly bind to the promoter of *Id3*, consistent with our observation that proliferating thymic iNKT cells express elevated levels of Id3 (33, 34). Thus, we hypothesize that E protein-controlled expression of Id3 establishes a negative feedback loop to limit excessive iNKT cell proliferation during maturation.

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 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.

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, while 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 *Il2rb* 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 PLZF^{high} NKT2 and ROR γ ⁺ 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 TBET⁺ NKT1 cells, Id3 is expressed by PLZF^{high} NKT2 cells. We propose, as recently suggested by Hu and colleagues, that Id3 may be a unique transcriptional regulator associated with the NKT2 sublineage (39). 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 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 Id3-GFP⁺ iNKT cells have higher expression levels of IL-17RB than Id3-GFP⁻ iNKT cells (unpublished observation and (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 and colleagues showed *Stat4* mRNA

levels were upregulated in NKT1 cells relative to NKT2 cells (5). 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, ROR γ ⁺ 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 ROR γ expression (28). However, with loss of Id3 and increased E protein transcriptional activity, we found only a slight increase in the frequency of the ROR γ ⁺ NKT subset, suggesting that E protein activity alone is not sufficient for commitment to this sublineage. Other transcription factors are likely to block ROR γ ⁺ NKT commitment in a dominant fashion. Indeed, the transcription factor Th-POK has recently been described to inhibit ROR γ 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, while 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 while 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 knock-out approach, led to increased iNKT cell number (31). Thus, it appears Id2 and Id3 play unique non-compensatory 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 PLZF^{high} NKT2 subset at the expense of the TBET⁺ 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 hyperreactivity (5, 40, 42). With abundant PLZF^{high} NKT2 cells, we would predict an increased susceptibility to AHR. Similarly, a greater frequency of PLZF^{high} 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 PLZF^{high} NKT2 sublineage (43, 44).

E protein transcription factors and their negative regulators the Id proteins have many important functions during lymphocyte development. We show here that E proteins control proliferation after positive selection and regulation of PLZF expression. Id2 and Id3 define and influence commitment to the TBET⁺ NKT1 and PLZF^{high} 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.

Acknowledgments

We would like to thank Drs. Engel, Best and Omilusik for critical reading of the manuscript.

References

1. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol.* 2013; 13:101–117. [PubMed: 23334244]
2. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol.* 2007; 25:297–336. [PubMed: 17150027]
3. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol.* 2011; 11:131–142. [PubMed: 21267014]
4. Montoya CJ, Pollard D, Martinson J, Kumari K, Wasserfall C, Mulder CB, Rugeles MT, Atkinson MA, Landay AL, Wilson SB. Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology.* 2007; 122:1–14. [PubMed: 17662044]
5. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, Yoshida H, Kubo M, Kawamoto H, Koseki H, Taniguchi M. Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol.* 2012; 10:e1001255. [PubMed: 22346732]
6. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nature immunology.* 2010; 11:197–206. [PubMed: 20139988]
7. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol.* 2005; 23:877–900. [PubMed: 15771592]
8. Lazarevic V, Zullo AJ, Schweitzer MN, Staton TL, Gallo EM, Crabtree GR, Glimcher LH. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nature immunology.* 2009; 10:306–313. [PubMed: 19169262]
9. Seiler MP, Mathew R, Liszewski MK, Spooner CJ, Barr K, Meng F, Singh H, Bendelac A. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nature immunology.* 2012; 13:264–271. [PubMed: 22306690]
10. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, Chua K, Eidson M, Kim HJ, Im JS, Pandolfi PP, Sant'Angelo DB. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature immunology.* 2008; 9:1055–1064. [PubMed: 18660811]
11. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, Lantz O, Bendelac A. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity.* 2008; 29:391–403. [PubMed: 18703361]
12. Dose M, Sleckman BP, Han J, Bredemeyer AL, Bendelac A, Gounari F. Intrathymic proliferation wave essential for V α 14⁺ natural killer T cell development depends on c-Myc. *Proc Natl Acad Sci U S A.* 2009; 106:8641–8646. [PubMed: 19423665]
13. Mycko MP, Ferrero I, Wilson A, Jiang W, Bianchi T, Trumpp A, MacDonald HR. Selective requirement for c-Myc at an early stage of V α 14i NKT cell development. *J Immunol.* 2009; 182:4641–4648. [PubMed: 19342639]
14. Allende ML, Zhou D, Kalkofen DN, Benhamed S, Tuymetova G, Borowski C, Bendelac A, Proia RL. SIP1 receptor expression regulates emergence of NKT cells in peripheral tissues. *FASEB J.* 2008; 22:307–315. [PubMed: 17785606]
15. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, Gapin L, Glimcher LH. T-bet regulates the terminal maturation and homeostasis of NK and V α 14i NKT cells. *Immunity.* 2004; 20:477–494. [PubMed: 15084276]
16. Ranson T, Voshchenrich CA, Corcuff E, Richard O, Laloux V, Lehuen A, Di Santo JP. IL-15 availability conditions homeostasis of peripheral natural killer T cells. *Proc Natl Acad Sci U S A.* 2003; 100:2663–2668. [PubMed: 12598649]
17. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Current opinion in immunology.* 2013; 25:161–167. [PubMed: 23402834]
18. Lee YJ, Holzappel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature immunology.* 2013

19. Murre C. Helix-loop-helix proteins and lymphocyte development. *Nature immunology*. 2005; 6:1079–1086. [PubMed: 16239924]
20. Cannarile MA, Lind NA, Rivera R, Sheridan AD, Camfield KA, Wu BB, Cheung KP, Ding Z, Goldrath AW. Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nature immunology*. 2006; 7:1317–1325. [PubMed: 17086188]
21. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, Li HS, Rivera RR, Lind KC, D'Cruz LM, Watowich SS, Murre C, Goldrath AW. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8(+) T cell subsets. *Nature immunology*. 2011
22. Miyazaki M, Rivera RR, Miyazaki K, Lin YC, Agata Y, Murre C. The opposing roles of the transcription factor E2A and its antagonist Id3 that orchestrate and enforce the naive fate of T cells. *Nature immunology*. 2011; 12:992–1001. [PubMed: 21857655]
23. D'Cruz LM, Lind KC, Wu BB, Fujimoto JK, Goldrath AW. Loss of E protein transcription factors E2A and HEB delays memory precursor formation during the CD8+ T-cell immune response. *Eur J Immunol*. 2012; 42:2031–2041. [PubMed: 22585759]
24. Vervakakis M, Boos MD, Bendelac A, Adams EJ, Pereira P, Kee BL. Inhibitor of DNA binding 3 limits development of murine slam-associated adaptor protein-dependent “innate” gammadelta T cells. *PLoS One*. 2010; 5:e9303. [PubMed: 20174563]
25. Monticelli LA, Yang Y, Knell J, D'Cruz LM, Cannarile MA, Engel I, Kronenberg M, Goldrath AW. Transcriptional regulator Id2 controls survival of hepatic NKT cells. *Proc Natl Acad Sci U S A*. 2009; 106:19461–19466. [PubMed: 19884494]
26. Alonzo ES, Gottschalk RA, Das J, Egawa T, Hobbs RM, Pandolfi PP, Pereira P, Nichols KE, Koretzky GA, Jordan MS, Sant'Angelo DB. Development of promyelocytic zinc finger and ThPOK-expressing innate gamma delta T cells is controlled by strength of TCR signaling and Id3. *J Immunol*. 2010; 184:1268–1279. [PubMed: 20038637]
27. Ueda-Hayakawa I, Mahlios J, Zhuang Y. Id3 restricts the developmental potential of gamma delta lineage during thymopoiesis. *J Immunol*. 2009; 182:5306–5316. [PubMed: 19380777]
28. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor HEB in thymocyte survival, Tcr rearrangement and the development of natural killer T cells. *Nature immunology*. 2010; 11:240–249. [PubMed: 20154672]
29. Jones ME, Zhuang Y. Acquisition of a functional T cell receptor during T lymphocyte development is enforced by HEB and E2A transcription factors. *Immunity*. 2007; 27:860–870. [PubMed: 18093538]
30. Pereira P, Boucontet L. Innate NKT $\gamma\delta$ and NKT $\alpha\beta$ cells exert similar functions and compete for a thymic niche. *Eur J Immunol*. 2012; 42:1272–1281. [PubMed: 22539299]
31. Li J, Wu D, Jiang N, Zhuang Y. Combined Deletion of Id2 and Id3 Genes Reveals Multiple Roles for E Proteins in Invariant NKT Cell Development and Expansion. *J Immunol*. 2013
32. Masson F, Minnich M, Olshansky M, Bilic I, Mount AM, Kallies A, Speed TP, Busslinger M, Nutt SL, Belz GT. Id2-mediated inhibition of E2A represses memory CD8+ T cell differentiation. *J Immunol*. 2013; 190:4585–4594. [PubMed: 23536629]
33. Schmitz R, Young RM, Ceribelli M, Jhavar S, Xiao W, Zhang M, Wright G, Shaffer AL, Hodson DJ, Buras E, Liu X, Powell J, Yang Y, Xu W, Zhao H, Kohlhammer H, Rosenwald A, Klun P, Muller-Hermelink HK, Ott G, Gascoyne RD, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Olgwang MD, Reynolds SJ, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Pittaluga S, Wilson W, Waldmann TA, Rowe M, Mbulaiteye SM, Rickinson AB, Staudt LM. Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature*. 2012; 490:116–120. [PubMed: 22885699]
34. Seitz V, Butzhammer P, Hirsch B, Hecht J, Gutgemann I, Ehlers A, Lenze D, Oker E, Sommerfeld A, von der Wall E, Konig C, Zinser C, Spang R, Hummel M. Deep sequencing of MYC DNA-binding sites in Burkitt lymphoma. *PLoS One*. 2011; 6:e26837. [PubMed: 22102868]
35. Doulatov S, Notta F, Rice KL, Howell L, Zelent A, Licht JD, Dick JE. PLZF is a regulator of homeostatic and cytokine-induced myeloid development. *Genes Dev*. 2009; 23:2076–2087. [PubMed: 19723763]

36. Li HS, Yang CY, Nallaparaju KC, Zhang H, Liu YJ, Goldrath AW, Watowich SS. The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. *Blood*. 2012; 120:4363–4373. [PubMed: 23033267]
37. Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, Ceredig R, Surh CD, Kronenberg M. Homeostasis of V alpha 14i NKT cells. *Nature immunology*. 2002; 3:966–974. [PubMed: 12244311]
38. Michel ML, Mendes-da-Cruz D, Keller AC, Lochner M, Schneider E, Dy M, Eberl G, Leite-de-Moraes MC. Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. *Proc Natl Acad Sci U S A*. 2008; 105:19845–19850. [PubMed: 19057011]
39. Hu T, Wang H, Simmons A, Bajana S, Zhao Y, Kovats S, Sun XH, Alberola-Illa J. Increased Level of E Protein Activity during Invariant NKT Development Promotes Differentiation of Invariant NKT2 and Invariant NKT17 Subsets. *J Immunol*. 2013
40. Terashima A, Watarai H, Inoue S, Sekine E, Nakagawa R, Hase K, Iwamura C, Nakajima H, Nakayama T, Taniguchi M. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med*. 2008; 205:2727–2733. [PubMed: 19015310]
41. Engel I, Zhao M, Kappes D, Taniuchi I, Kronenberg M. The transcription factor Th-POK negatively regulates Th17 differentiation in Valpha14i NKT cells. *Blood*. 2012; 120:4524–4532. [PubMed: 23034280]
42. Stock P, Lombardi V, Kohlrantz V, Akbari O. Induction of airway hyperreactivity by IL-25 is dependent on a subset of invariant NKT cells expressing IL-17RB. *J Immunol*. 2009; 182:5116–5122. [PubMed: 19342692]
43. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology*. 2011; 140:1756–1767. [PubMed: 21530742]
44. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity*. 2002; 17:629–638. [PubMed: 12433369]

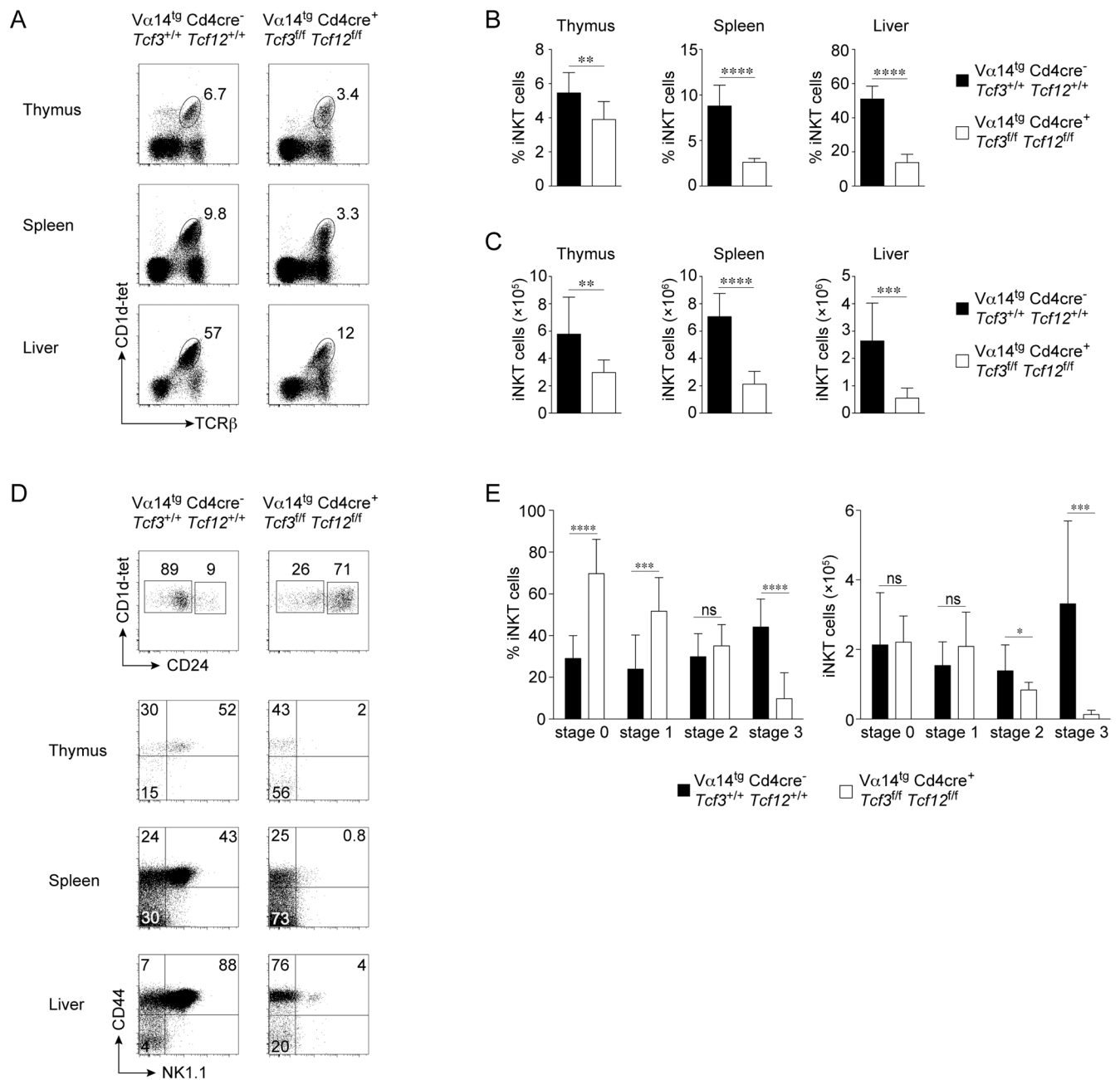


Figure 1. Loss of E protein expression impairs iNKT cell maturation

$Tcf3^{+/+}Tcf12^{+/+}Cd4cre^{-}$ or $Tcf3^{fl/fl}Tcf12^{fl/fl}Cd4cre^{+}$ mice expressing a $V\alpha 14$ -Ja18 TCR transgene were characterized. (A) Flow cytometric analysis of iNKT cell frequency in $V\alpha 14$ -Ja18tg $^{+}Tcf3^{+/+}Tcf12^{+/+}Cd4cre^{-}$ and $V\alpha 14$ -Ja18tg $^{+}Tcf3^{fl/fl}Tcf12^{fl/fl}Cd4cre^{+}$ lymphocytes from indicated tissues. Numbers indicate percentage of cells within the drawn gate. (B) Average frequency or (C) absolute number (\pm SEM) of CD1d-tet $^{+}TCR\beta^{+}$ gated iNKT cells from the indicated genotype and tissue. (D) Flow cytometric analysis of CD1d-tet $^{+}CD24^{+}$ expression in the thymus and CD44 $^{+}NK1.1^{+}$ expression by gated iNKT lymphocytes from the indicated genotype in the indicated tissue. (E) Average frequency and absolute number (\pm SEM) of maturation stages as defined by CD1d-tet and CD24 or CD44 and NK1.1 expression on CD1d-tet $^{+}TCR\beta^{+}$ gated iNKT cells from the indicated genotype in

the thymus. Stage 0 cells are defined by CD1d-tet⁺CD24⁺ expression. Gated CD1d-tet⁺CD24⁻ cells define stages 1, 2 and 3. Graphs are the average of 11 mice from 8 independent experiments. Statistical significance was determined using unpaired two-tailed t test, ns = not significant, *, P < 0.05, **, P < 0.005, ***, P < 0.0005, ****, P < 0.0001.

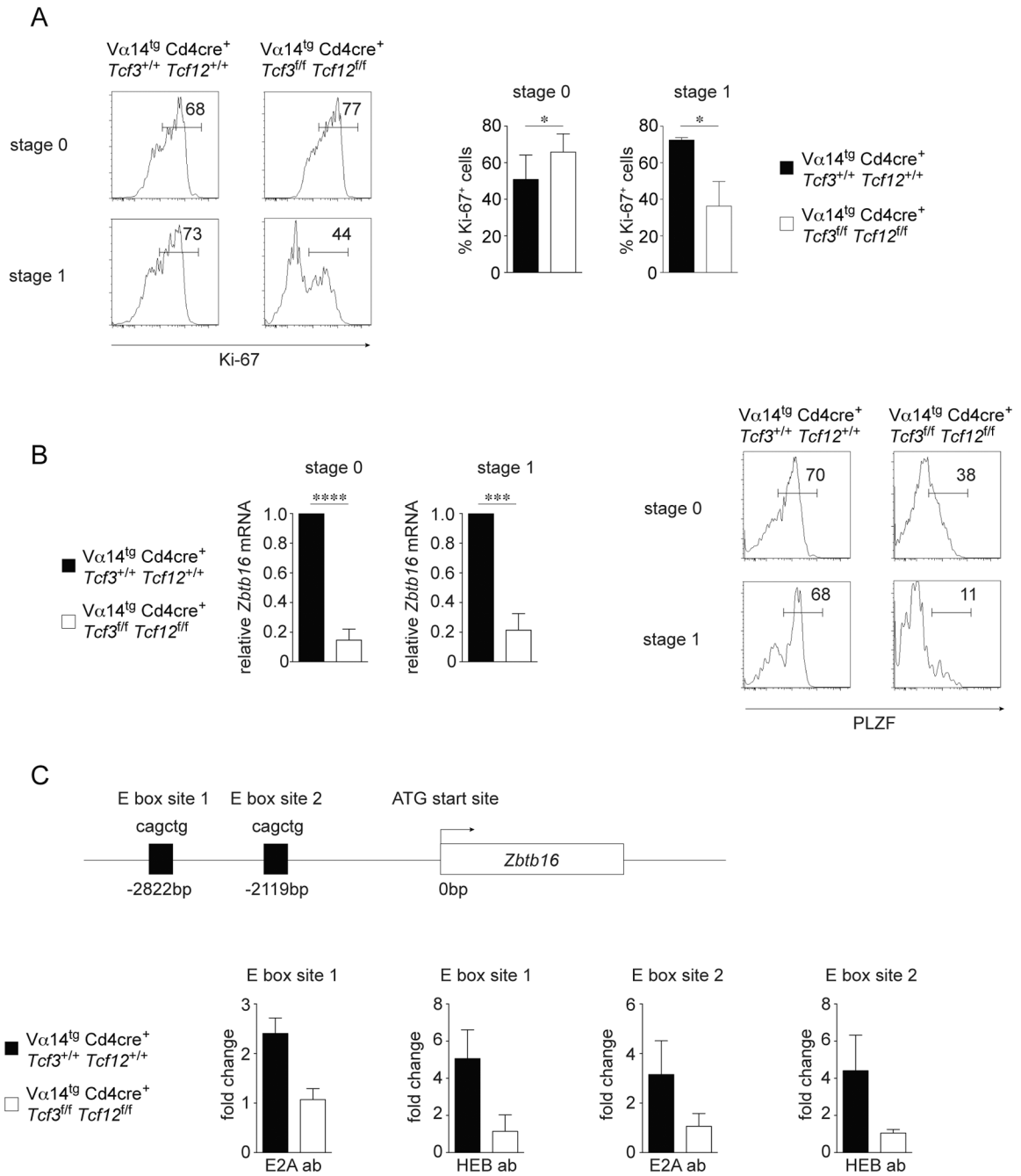


Figure 2. E proteins regulate proliferation and PLZF expression in thymic iNKT cells
 (A) Ki-67 expression by stage 0 and stage 1 CD1d-tet⁺TCRβ⁺ gated iNKT thymocytes from the indicated genotypes. Numbers indicate percentage Ki-67⁺ cells within the gate. Bar graphs indicate average frequency (±SEM) of CD1d-tet⁺TCRβ⁺ gated iNKT Ki-67⁺ cells at stage 0 and 1. Graphs are an average of 6 mice from 3 individual experiments. Statistical significance was determined using unpaired two-tailed t test, *, P < 0.05. (B) Bar graphs indicate relative expression of *zbtb16* mRNA by stage 0 and 1 CD1d-tet⁺TCRβ⁺ gated iNKT cells determined by qPCR. Data are normalized to Vα14-Jα18tg⁺Tcf3^{+/+}Tcf12^{+/+}Cd4cre⁻ cells and are the average of 3 individual samples from 3 independent experiments. Histogram plots indicate PLZF expression by stage 0 and 1 CD1d-tet⁺TCRβ⁺ CD1d-

tet⁺TCRβ⁺ gated iNKT cells from the indicated genotypes. Numbers indicate percentage of PLZF^{high} cells within the gate. Data are representative of 3 independent experiments with $n = 1 - 3$ mice per group. Statistical significance was determined using unpaired two-tailed t test, **, $P < 0.005$, ****, $P < 0.0001$. (C) The *zbtb16* promoter is a direct target of E2A and HEB. Schematic indicating 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⁺TCRβ⁺CD24⁺) and chromatin was precipitated with anti-E2A, anti-HEB or control IgG antibodies. The relative amounts of input or precipitated DNA containing the indicated E box sites were determined by qPCR. Fold change of Vα14-Jα18tg⁺Tcf3^{+/+}Tcf12^{+/+}Cd4cre⁻ pull-down over Vα14-Jα18tg⁺Tcf3^{f/f}Tcf12^{f/f}Cd4cre⁺ control are graphed and are an average of sorted cells from 3 mice for Vα14-Jα18tg⁺Tcf3^{+/+}Tcf12^{+/+}Cd4cre⁻ and 2 mice for Vα14-Jα18tg⁺Tcf3^{f/f}Tcf12^{f/f}Cd4cre⁺.

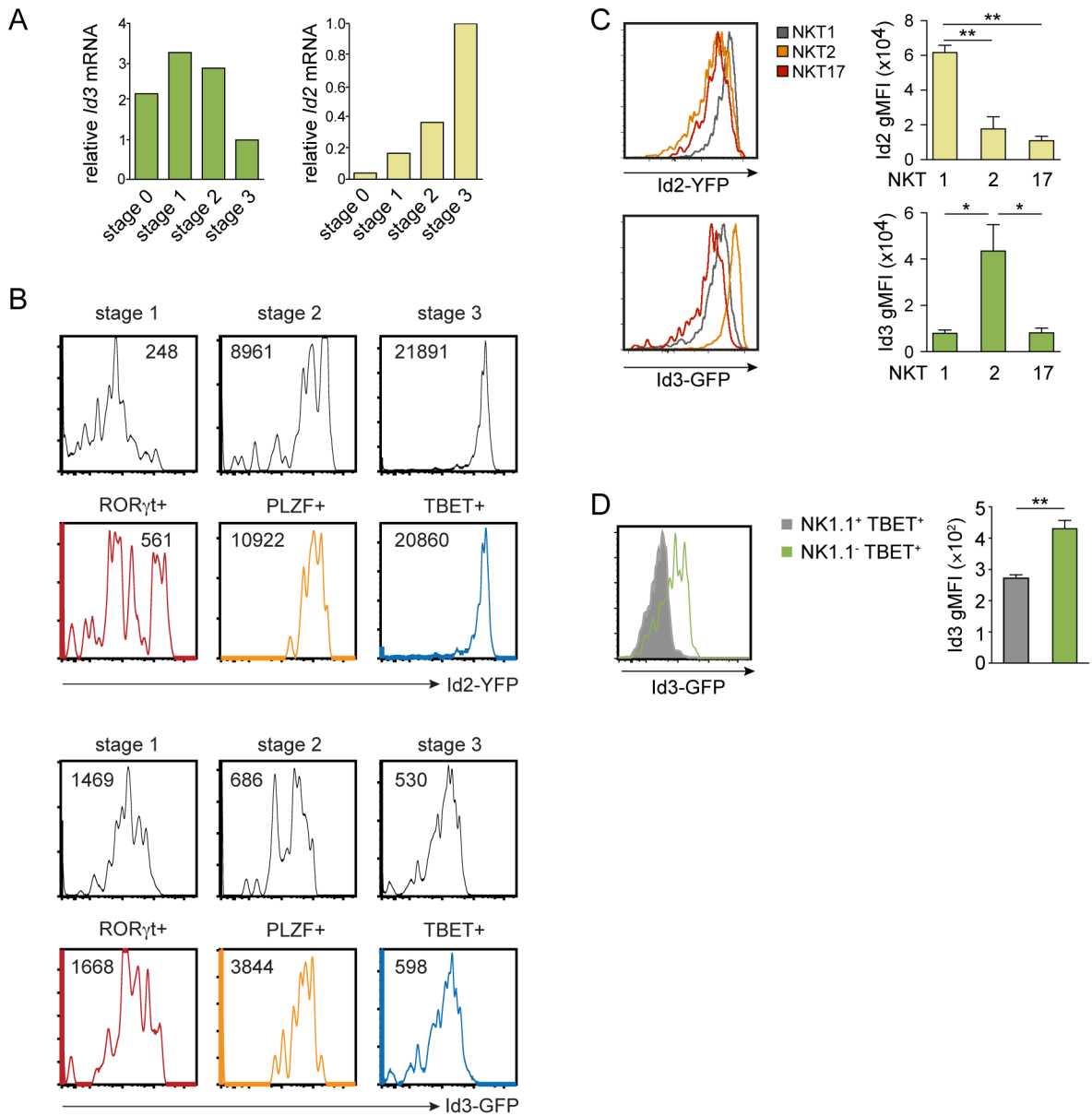


Figure 3. Id2 and Id3 expression define iNKT cell developmental stages and sublineages in the thymus

(A) Relative Id3 and Id2 mRNA expression levels by sorted wild-type thymic CD1d-tet⁺TCRβ⁺ iNKT cells. Relative expression indicated is normalized to stage 3 mRNA levels. Data are representative of three independent experiments. (B) Histograms showing Id3-GFP and Id2-YFP reporter expression by CD1d-tet⁺TCRβ⁺ gated iNKT cells at stage 1 (CD24⁻CD44⁻NK1.1⁻), stage 2 (CD24⁻CD44⁺NK1.1⁻) and stage 3 (CD24⁺CD44⁺NK1.1⁺) or by iNKT cells expressing TBET, PLZF or RORγt in the thymus. Numbers in histograms indicate mean fluorescence intensity (MFI). Data are representative of 3 mice per group from 3 independent experiments. (C) Histograms showing Id2-YFP and Id3-GFP expression in gated CD1d-tet⁺TCRβ⁺ TBET⁺ NKT1, PLZF^{high} NKT2 and RORγt⁺ NKT17 cells respectively. Bar graphs indicate average Id2-YFP and Id3-GFP MFI of indicated sublineages. (E) Histogram showing Id3-GFP expression of NK1.1⁻ TBET⁺ (green line) and NK1.1⁺ TBET⁺ (grey line) cells. Bar graph indicates average Id3-GFP MFI of indicated cell

type. Data are average (\pm SEM) of 3 mice from 3 independent experiments. Statistical significance was determined using one-way ANOVA and Bonferroni post-hoc test, *, $P < 0.05$, **, $P < 0.005$.

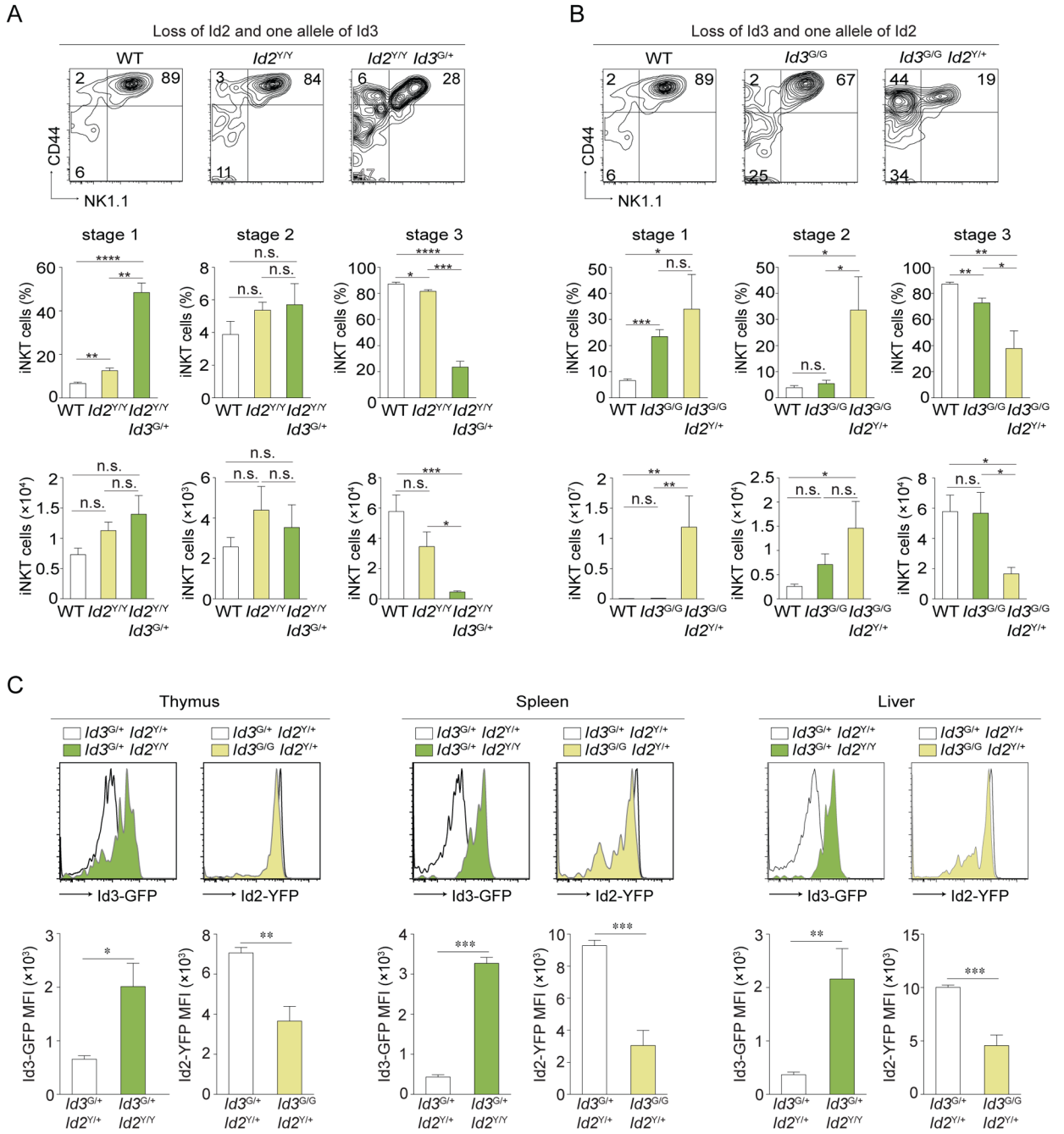


Figure 4. Compensation by Id2 and Id3 as iNKT cells differentiate in the thymus

Flow cytometric analysis showing CD44⁺NK1.1⁺ expression by CD1d-tet⁺TCRβ⁺ gated iNKT cells from (A) wild type (WT), Id2-deficient (*Id2*^{Y/Y}) and Id2-deficient Id3-heterozygote (*Id2*^{Y/Y}*Id3*^{G/+}) fetal liver chimeras and (B) wild type (WT), Id3-deficient (*Id3*^{G/G}) and Id3-deficient Id2-heterozygote (*Id3*^{G/G}*Id2*^{Y/+}) fetal liver chimeras. 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 4 fetal liver chimeras per group, representative of 2 independent experiments. Statistical significance was determined

using one-way ANOVA or unpaired two-tailed t test, *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$, ****, $P < 0.0001$.

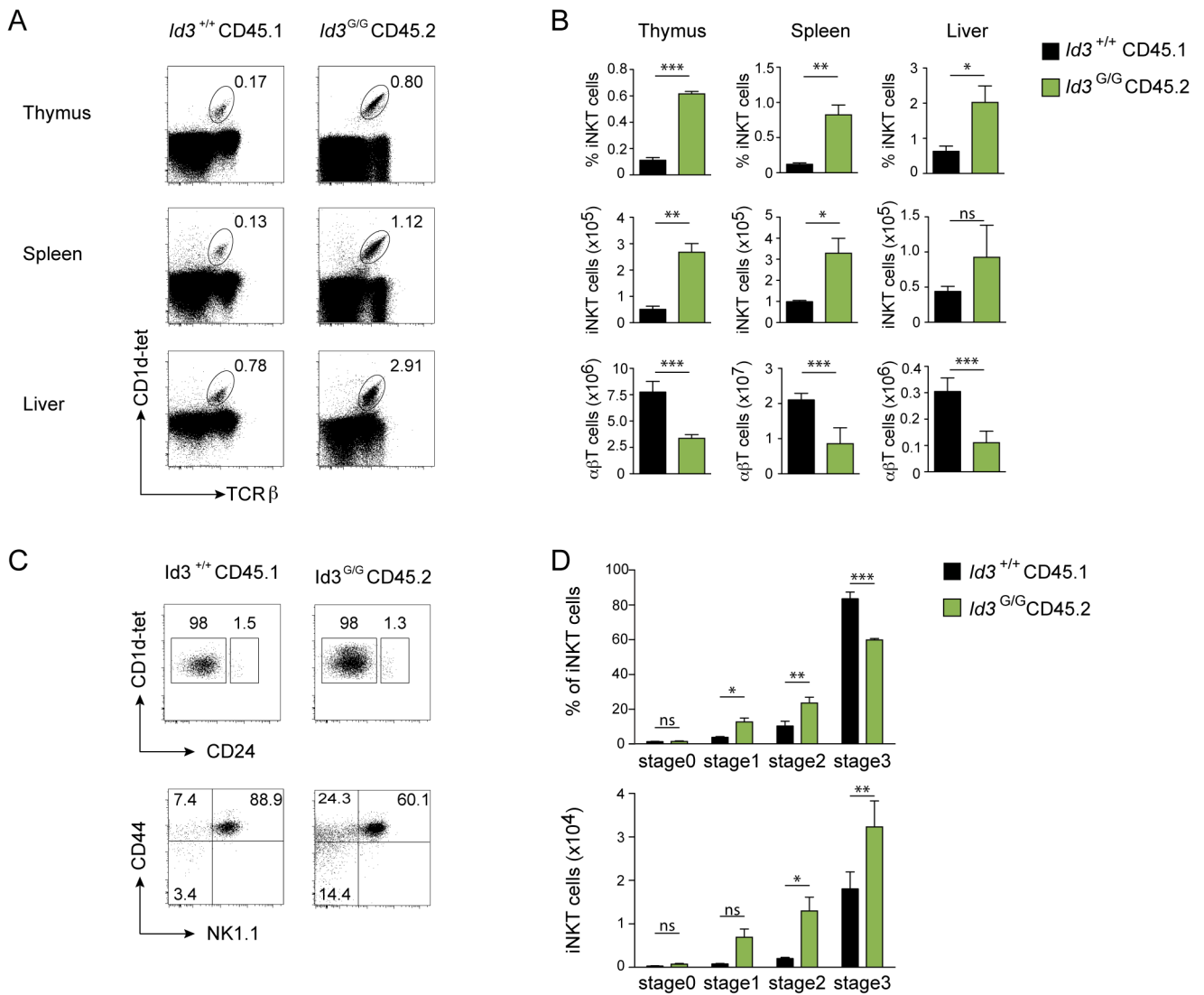


Figure 5. Id3 controls iNKT cell accumulation and maturation

Mixed bone marrow chimeras were generated by transfer of a 2:1 mix of CD45.1 *Id3*^{+/+} with CD45.2 *Id3*^{G/G} bone marrow into irradiated recipients. (A) Flow cytometry plots showing iNKT cell frequency in wild-type *Id3*^{+/+} and *Id3*-deficient *Id3*^{G/G} lymphocytes from indicated tissues. Numbers indicate percentage of cells within the gate. (B) Average frequency and absolute number (\pm SEM) of *Id3*^{+/+} and *Id3*^{G/G} CD1d-tet⁺TCR β ⁺ iNKT cells or conventional $\alpha\beta$ T cells from indicated tissues. (C) CD1d-tet⁺CD24⁺ and CD44⁺NK1.1⁺ expression by CD1d-tet⁺TCR β ⁺ gated *Id3*^{+/+} and *Id3*^{G/G} iNKT thymocytes assessed by flow cytometry. Numbers indicate percentage of cells within the gate. (D) Average frequency and absolute number (\pm SEM) of maturation stages for CD1d-tet⁺TCR β ⁺ gated *Id3*^{+/+} and *Id3*^{G/G} iNKT thymocytes. Data are average (\pm SEM) of 3 mice, representative of 3 independent experiments with $n = 3 - 4$ mice. Statistical significance was determined using unpaired two-tailed t test, ns = not significant, *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$.

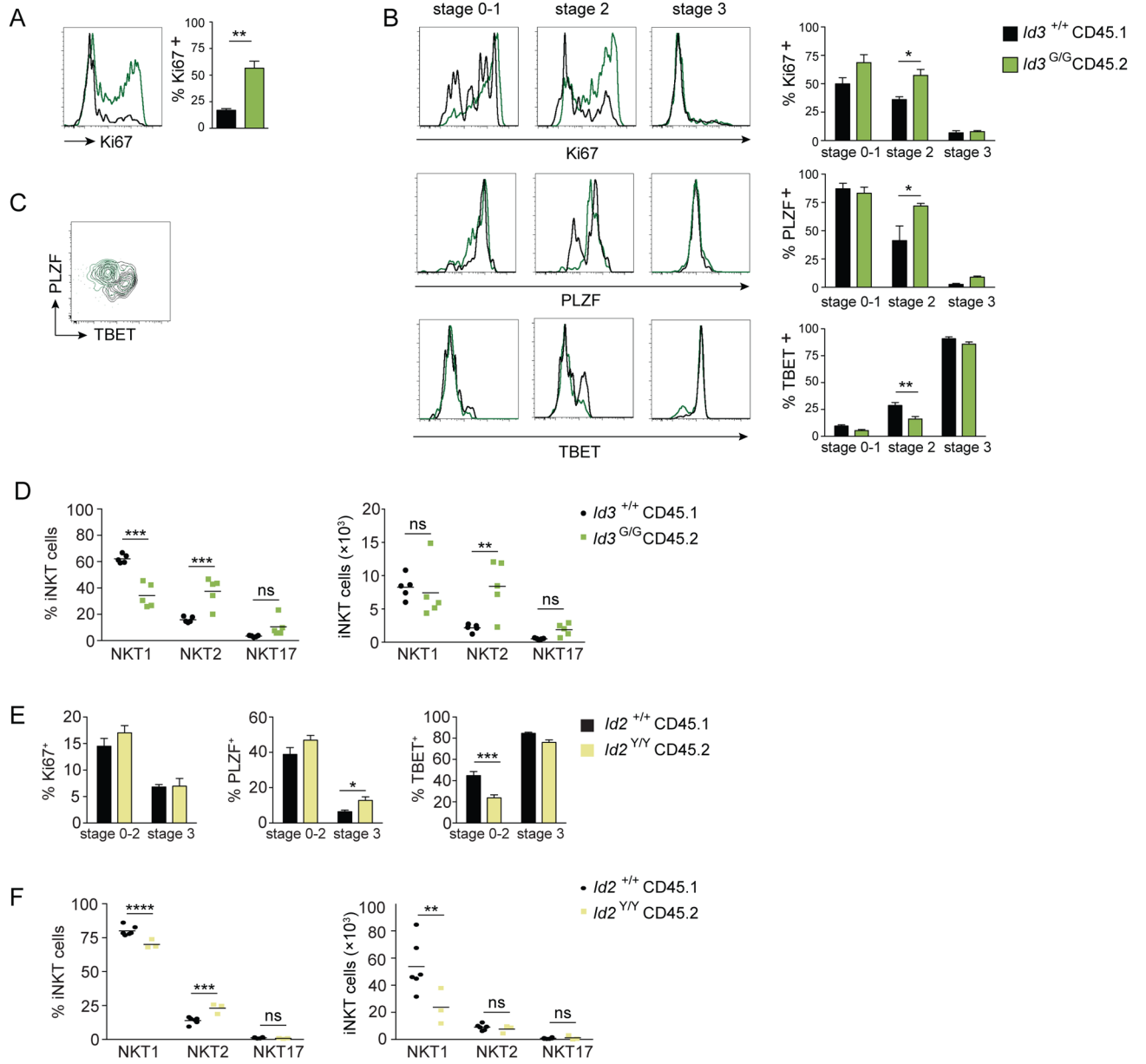


Figure 6. Id3 regulates proliferation and sublineage commitment in iNKT cells

Mixed bone marrow chimeras were generated by transfer of a 2:1 mix of *Id3*^{+/+} to *Id3*^{G/G} bone marrow to irradiated recipients. (A) Histograms showing Ki-67 staining by gated wild-type (*Id3*^{+/+}) (black line) and *Id3*-deficient (*Id3*^{G/G}) (green line) iNKT cells from the thymus. Bar graphs indicate average frequency (±SEM) of Ki-67⁺ iNKT cells. (B) Histograms showing Ki-67, PLZF and TBET expression by CD1d-tet⁺TCRβ⁺ gated wild-type (*Id3*^{+/+}) (black line) and *Id3*-deficient (*Id3*^{G/G}) (green line) iNKT cells at stage 0–1, stage 2 and stage 3 in the thymus. Bar graphs indicate frequency of Ki-67⁺, PLZF^{high} and TBET⁺ cells by CD1d-tet⁺TCRβ⁺ gated *Id3*^{+/+} and *Id3*^{G/G} iNKT thymocytes at indicated

developmental stages. Data are average (\pm SEM) of 3 mice, representative of 3 independent experiments. (C) Flow cytometry plot indicating PLZF and TBET expression by wild-type (*Id3*^{+/+}) (black) and *Id3*-deficient (*Id3*^{G/G}) (green) iNKT thymocytes. (D) Graphs of relative frequency and absolute number of TBET⁺ NKT1, PLZF^{high} NKT2 and ROR γ ⁺ NKT17 cells of total gated *Id3*^{+/+} and *Id3*^{G/G} iNKT thymocytes. Data are average of 5 mice, representative of 3 independent experiments. (E) Bar graphs indicate frequency of Ki-67⁺, PLZF⁺ and TBET⁺ cells by CD1d-tet⁺TCR β ⁺ gated *Id2*^{+/+} and *Id2*^{Y/Y} iNKT thymocytes at indicated developmental stages. Data are average (\pm SEM) of 6 mice, representative of 2 independent experiments. (F) Graphs of relative frequency and absolute number of TBET⁺ NKT1, PLZF^{high} NKT2 and ROR γ ⁺ NKT17 cells of total gated *Id2*^{+/+} and *Id2*^{Y/Y} iNKT thymocytes. Data are average of 6 mice (*Id2*^{+/+}) and 3 mice (*Id2*^{Y/Y}), representative of 3 independent experiments. Statistical significance was determined using Mann Whitney U test and unpaired two-tailed t test where applicable, ns = not significant, *, P < 0.05, **, P < 0.005, ***, P < 0.0005.