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Combined Deletion of Id2 and Id3 Genes Reveals Multiple Roles for E Proteins in Invariant NKT Cell Development and Expansion

Jia Li,* Di Wu,†,‡ Ning Jiang,†,‡ and Yuan Zhuang*

The invariant NKT (iNKT) cells represent a unique group of \(\alpha\)\(\beta\) T cells that have been classified based on their exclusive usage of the invariant Vo14J\(a\)18 TCR\(\alpha\)-chain and their innate-like effector function. Thus far, the transcriptional programs that control Vo14J\(a\)18 TCR\(\alpha\) rearrangements and the population size of iNKT cells are still incompletely defined. E protein transcription factors have been shown to play necessary roles in the development of multiple T cell lineages, including iNKT cells. In this study, we examined E protein functions in T cell development through combined deletion of genes encoding E protein inhibitors Id2 and Id3. Deletion of Id2 and Id3 in T cell progenitors resulted in a partial block at the pre-TCR selection checkpoint and a dramatic increase in numbers of iNKT cells. The increase in iNKT cells is accompanied with a biased rearrangement involving Vo14 to J\(a\)18 recombination at the double-positive stage and enhanced proliferation of iNKT cells. We further demonstrate that a 50% reduction of E proteins can cause a dramatic switch from iNKT to innate-like \(\gamma\)\(\delta\) T cell fate in Id2- and Id3-deficient mice. Collectively, these findings suggest that Id2- and Id3-mediated inhibition of E proteins controls iNKT development by restricting lineage choice and population expansion. The Journal of Immunology, 2013, 191: 5052–5064.

Development of T cells in the thymus generates multiple types of T cells that belong to different lineages, defined primarily by the types of TCRs they use. The \(\alpha\)\(\beta\) T lineage is specified after expression of the pre-TCR composed of the TCR\(\beta\)-chain and the invariant pre-TCR\(\alpha\)-chain. These precursor T cells then undergo proliferative expansion before rearranging the TCR\(\alpha\)-chain. Upon generation of the \(\alpha\)\(\beta\) TCR, most \(\alpha\)\(\beta\) T cells differentiate into CD8 cytolytic or CD4 helper lineages based on their ability to recognize peptide Ags presented by either the MHC class I or class II molecules, respectively. A small fraction of \(\alpha\)\(\beta\) T cells form the NKT cell lineage owing to their TCR selection by lipid Ags presented by CD1d, an MHC-like molecule (1, 2). NKT cells represent a distinct effector group that is capable of providing diverse and fast effector functions and thus is also classified as innate-like T cells (3).

A large fraction of NKT cells use a canonical TCR\(\alpha\)-chain resulting from V\(a\)14 to J\(a\)18 rearrangement and are thus named invariant NKT (iNKT) cells (4). The remaining NKT cells, referred to as type II NKT cells, also show highly restricted V\(a\)-Ja usages and recognize lipid Ags different from those recognized by iNKT cells (5–8). iNKT cells share the same developmental history with the rest of \(\alpha\)\(\beta\) T cells up to the double-positive (DP) stage (DP for CD4 and CD8 expression), where the TCR\(\alpha\) gene rearranges. Expression and selection of an appropriate TCR\(\alpha\)-chain at this stage have been shown to provide the driving force in iNKT cell development (9–11). Most of our understanding of iNKT lineage development is based on events during and after TCR\(\alpha\) gene expression at the DP stage. It is not entirely clear whether the highly restricted V\(a\)-Ja usage for NKT cells is simply a result of TCR-mediated selection or additional regulation prior to TCR selection (12, 13).

E proteins and their inhibitors Id proteins have been shown to play important roles at the pre-TCR, the \(\gamma\)\(\delta\)TCR, and the \(\alpha\)\(\beta\)TCR checkpoints (14, 15). E proteins are basic-HLH motif-containing transcription factors, which bind E-box DNA sequences as dimers (16). Id proteins inhibit E protein function through competitive dimerization with E proteins, thereby preventing E proteins from binding to DNA (17). Two E protein genes, E2A and HEB, and two Id genes, Id3 and Id2, have been shown to be involved in producing E proteins and Id proteins, respectively, during T cell development (18). Although removal of a single E protein gene only resulted in partial defects in T cell development, deletion of both E2A and HEB genes in the early stages of T cell development resulted in nearly complete block in \(\alpha\)\(\beta\) lineage development and severe impairment in \(\gamma\)\(\delta\) lineage development (19). Conditional deletion of E2A and HEB at the DP stage with CD4-Cre also demonstrated an essential role for E proteins in CD4 lineage and iNKT lineage development (20–22). In contrast to E protein gene knockout, deletion of Id3 early in T cell development resulted in a significant increase in \(\gamma\)\(\delta\) lineage T cells (23), although this increase is almost exclusively restricted to innate-like \(\gamma\)\(\delta\) T cells expressing the V\(\gamma\)1.1V\(\delta\)6.3 TCRs (24). The enhanced \(\gamma\)\(\delta\) lineage development has been attributed to elevated levels of E protein activities because deletion of Id3 and E2A together can correct the innate \(\gamma\)\(\delta\) T phenotype. These genetic studies clearly demonstrated that E protein dosage plays an important role in influencing the fate choice between the \(\gamma\)\(\delta\) and \(\alpha\)\(\beta\) lineages at the pre-TCR and...
γδTCR checkpoints, reminiscent of E protein functions at the αβTCR checkpoint (14, 25).

Given that Id2 has been shown to collaborate with Id3 in regulating the TCR checkpoint (22), it is speculated that Id2 could also collaborate with Id3 in regulating the pre-TCR and γδTCR checkpoints. In this study, we used Lck-Cre (26) to delete both Id2 and Id3 at the pre-TCR and γδTCR checkpoints. Deletion of both Id2 and Id3 resulted in a partial block at the pre-TCR checkpoint and increased production of innate γδ T cells, suggesting opposing roles for Id genes in regulating the γδ lineage and the innate γδ lineage. More importantly, analysis of Id2 and Id3 double-deficient animals also revealed a novel role for Id2 and Id3 in regulating the development and expansion of iNKT cells. The mutant mice showed a dramatic increase in numbers of iNKT cells. A biased rearrangement involving Vα14-Jα18 was detected in preselecting DP cells, indicating a role for Id proteins in regulating Vα proteins before being submitted to the High/V-QUEST search engine (32). A TCR to the FAST format (31) on a Galaxy platform (http://galaxyproject.org/) also collaborated with Mac-1, B220, and NK1.1 Abs was included in staining as the dump role for Id genes in regulating the TCR checkpoint (22), it is speculated that Id2 and Id3 double-deficient mice showed a small fraction of TCR αβ T cells expressed TCR αβ prior to CD1d-mediated selection. Results presented in this study further suggest a dosage-dependent mechanism for Id genes in repressing the fate of innate-like γδ T cells versus iNKT cells during T cell development.

Materials and Methods

Mice

The Lck-Cre transgenic allele (26) and flox alleles for the Id3 (27), Id2 (28), E2A (26), and HEB (19) genes have been previously described. Mice used in this study have been maintained on 129sv and C57BL/6 mixed background in a specific pathogen-free facility managed by Duke University Division of Laboratory Animal Resources. All procedures have been conducted according to protocols approved by the Institutional Animal Care and Use Committee.

Cell staining and flow cytometry

BrdU staining (BD Biosciences) was performed according to the manufacturer’s protocol. Intracellular staining was performed after 2% formaldehyde fixation and 0.5% saponin permeabilization. Staining for promyelocytic leukemia zinc finger (PLZF) Ab (provided by Dr. Derek Sant’Angelo) was carried out using a Foxp3 staining buffer set (eBioscience). CD1d tetramers (CD1deto) with or without loaded PBS57 Ag were obtained from the Tetramer Facility of the National Institutes of Health. CD1d staining was done in dark for 30 min at room temperature before Ab staining of other cell surface markers. FACS was performed on a FACSCanto II (BD Biosciences). Doubles and dead cells were gated out before data analysis. Data were analyzed with FlowJo software (Tree Star).

In vitro OP9-DL1 culture

FACS sorting of double-negative (DN) cells was performed after Dynabead biotin binder (Invitrogen) treatment to deplete CD4 single-positive (SP), CD8 SP, and DP cells. The remaining cells were stained with CD44, CD25, TCRγδ, and CD27 Abs. A mixture of CD4, CD8, Gr-1, Mac-1, B220, and NK1.1 Abs was included in staining as the dump channel to eliminate non-T cells and any residual CD4 SP, CD8 SP, and DP cells. Cells were cultured in MEM-α medium (10% FBS, penicillin/streptomycin, and 5 ng/ml IL-7 for all cultures and additional 5 ng/ml Flt3 ligand for the DN3 culture) on OP9-DL1 cell coated plates. Lymphocytes were harvested and analyzed by FACS at the specified time points.

V(D)J rearrangement analysis

cDNA from FACS-sorted thymocyte fractions were prepared as described (22). For Vα14-Jα18 usage in CD1d+sorted iNKT cells, cDNAs were amplified with primers as described (21, 29). RT-PCR products were subcloned into TOPO TA vector (Invitrogen) and sequenced. Jα gene segment usage was determined using the V-QUEST search program (30) and verified by manual check. For Jα repertoire analysis of DP cells, a Vγ6–9 or Vα14-specific primer and a Co primer were used to generate libraries for Ion Torrent high-throughput sequencing. Data were converted to the FAST format (31) on a Galaxy platform (http://galaxyproject.org/) before being submitted to the High/V-QUEST search engine (32). A TCRβ V-D-J rearrangement assay was performed with primers VB5-3’ and JB2.7-3’ according to the protocol described previously (19). Genomic DNA was extracted from FACS-sorted cell fractions.

Results

Conditional removal of Id2 and Id3 with Lck-Cre impairs T cell development

We hypothesized that Id2 may play a redundant role and functionally compensate for the loss of function of Id3 at the pre-TCR checkpoint. To test this hypothesis, we used Lck-Cre to drive conditional deletion of Id3 and Id2 before the pre-TCR and γδTCR checkpoints. PCR analysis of fractionated thymocytes demonstrated that Lck-Cre–mediated deletion of the Id2 and Id3 floxed alleles started at the DN3 stage and achieved near completion by the DP stage (Supplemental Fig. 1). Mice carrying the Lck-Cre transgene or the floxed alleles alone did not show any phenotype (Supplemental Fig. 2A). Lck-Cre–induced Id2 deletion alone did not produce any developmental abnormalities, whereas deletion of both copies of Id3 plus one copy of Id2 resulted in a dramatic increase in γδ T cells and a reduction in αβ T cells (Fig. 1A, bottom panel), a result similar to a previous report of Id3 knockout mice (23). Deletion of both Id2 and Id3 with Lck-Cre (referred to as L-DKO hereafter) resulted in a phenotype significantly different from these control groups. The total thymic cellularity of L-DKO mice was reduced to an average of 36% (among 1.5-mo-old young adults) or 43% (at the weaning age) in comparison with age-matched Cre− control mice (Fig. 1B).

consistent with an earlier study involving CD4−Cre–mediated deletion of Id2 and Id3 at the DP stage, Lck-Cre–mediated deletion of Id2 and Id3 at the DN stage also resulted in a complete block in CD8 lineage development (22) (Fig. 1A, upper panel). L-DKO mice also exhibited a significant increase in numbers and percentages of CD4−CD8− (DN) cells (Fig. 1A, middle panel). Further analysis showed that the increase in DN cells could be attributed mostly to a change in the CD4−CD8−CD25−CD44− (DN4) fraction (from 6.7 ± 1.1 × 103 in Cre+ mice to 26.5 ± 5.2 × 103 in L-DKO mice; Supplemental Fig. 2B). The absolute numbers of DN3 and DP cells in L-DKO mice were decreased to approximately half of the Cre− controls (Supplemental Fig. 2B, 2C). Collectively, these results indicated that Lck-Cre induced deletion of Id2 and Id3 perturbed T cell development at multiple stages, including the DN3 stage.

Accumulation of CD4−CD8−TCRβlo cells occurs during neonatal life

Further examination of L-DKO thymocytes showed that their CD4−CD8− cells expressed TCRβ at a level lower than that of CD4 SP in Cre− controls (Fig. 1C). TCRβlo cells were also observed among most CD4−CD8− cells and peripheral CD4 T cells in L-DKO mice. Both L-DKO and Id2+/Id3f/f/Lck-Cre− mice also showed a small fraction of TCRβ+ cells among the CD4 T cells in the thymus and periphery. A separate FACS analysis showed that these TCRβ+ CD4−CD8− T cells were innate-like γδ T cells as reported in Id3 knockout mice (24). To uncover the primary developmental defects and to limit the potential cross-regulation from effector T cells generated in the mutant mice, we switched to examination of neonatal animals (Fig. 1D). Among day 3 neonates, CD4 cells were not detected in L-DKO mice, indicating a developmental block in the transition from DP to CD4 SP. CD4−CD8−TCRβ+ T cells were also absent at this early stage. In contrast, a dramatic increase in CD4−CD8−TCRβ+ T cells and γδ T cells became apparent 10 d after birth concomitant with the appearance of CD4 cells. By day 20 (weaning age), both CD4−CD8− TCRβ+ T cells and CD4 cells exhibited a further increase in percentages relative to other populations in the thymus of L-DKO mice. CD4 cells in the mutant mice expressed a lower level of TCRβ in comparison with the CD4 cells in the Cre− control mice.
FIGURE 1. Early T cell development is perturbed by Lck-Cre–induced knockout of Id2 and Id3. (A) Total thymocytes were analyzed in two-dimensional plots of either CD4 and CD8a (top panel) or TCRβ and TCRδ (bottom panel). The DN fractions (defined by CD4<sup>−</sup>CD8<sup>−</sup>NK1.1<sup>−</sup>B220<sup>−</sup>Gr-1<sup>−</sup>Mac-1<sup>−</sup>) of thymocytes were displayed by CD44 and CD25 expression (middle panel). Samples are from 2-mo-old Id2<sup>+/+</sup>Id3<sup>+/+</sup>LckCre<sup>−</sup>, Id2<sup>f/f</sup>Id3<sup>+/+</sup>LckCre<sup>+</sup>, Id2<sup>+/f</sup>Id3<sup>f/f</sup>LckCre<sup>+</sup>, and Id2<sup>f/f</sup>Id3<sup>f/f</sup>LckCre<sup>+</sup> mice as indicated on the top of each column. Percentages of cells in each quadrant are displayed. (B) Cell counts of total thymocytes in 1.5-mo-old and 20-d-old mice. The mean value for 1.5-mo-old Cre<sup>−</sup> control mice is 2.4 ± 0.2 × 10<sup>8</sup> (n = 7), and for Cre<sup>+</sup> L-DKO mice is 0.85 ± 0.13 × 10<sup>8</sup> (n = 8). The p value of the t test between the two groups is 0.0001. The mean value for 20-d-old Cre<sup>−</sup> control mice is 13 ± 1.1 × 10<sup>7</sup> (n = 5), and for Cre<sup>+</sup> L-DKO is 5.6 ± 1.2 × 10<sup>7</sup> (n = 5). The p value of the t test between the two groups is 0.0017. (C) Expression level of TCRβ among indicated cell fractions isolated from thymus, spleen, and lymph nodes. (D) FACS analysis of day 3, 10, and 20 neonatal Cre<sup>−</sup> and Cre<sup>+</sup> pairs at each time point. (E) Cre<sup>−</sup> (red) and Cre<sup>+</sup> (blue) samples from day 20 shown in (D) were further analyzed in an overlay histogram to display TCRβ expression in DP and CD4 SP cells.
Based on these findings, we chose 20-d-old animals to further dissect T cell development in L-DKO mice.

**A partial block at the pre-TCR checkpoint**

Given that Lck-Cre initiates Id2 and Id3 deletion at the DN3 stage, we first evaluated the effect of Id deletion on pre-TCR selection. We used CD27 to separate DN3 cells into DN3a (CD27lo) and DN3b (CD27hi) fractions (Fig. 2A). CD27 upregulation is tightly correlated with pre-TCR selection among DN3 cells (33). Analysis of L-DKO mice showed that the percentage of DN3b cells within the DN3 fraction was reduced to approximately one-half of that in Cre+ control littermates (Fig. 2B). To determine whether this block in pre-TCR selection was due to any major perturbations of TCRβ gene rearrangement, we examined TCRβ gene usage based on V-D-J rearrangements (19). A random pattern of Jβ usage was detected among DN3 and DN4 cells in Cre+ mice and DN3 cells from L-DKO mice (Fig. 2C). This result indicated a relatively normal TCRβ usage among DN3 cells in L-DKO mice. However, both CD4+CD8− TCRβ+ and CD4−CD8+ TCRβ− cells in L-DKO mice showed a perturbed pattern of D-J usage.

To further evaluate the efficiency of pre-TCR selection, we tested the differentiation capability of DN3 cells in an OP9-DL1 culture system (34). DN3 cells from both Cre+ control and L-DKO samples expanded dramatically and differentiated into the DP stage within 6 d in culture (Fig. 2D, 2E). However, DN3a cells from L-DKO mice progressed from DN to DP in a slower kinetics in comparison with the Cre+ controls (Fig. 2D). The kinetic difference between these two genotype groups was also evident among the sorted DN3b cells (Fig. 2E), which represents cells having undergone β-selection (33). Collectively, these data indicate that

![Figure 2](http://www.jimmunol.org/)
id Proteins Restrict iNKT Lineage Development

Id proteins restrict iNKT lineage development

CD4+CD8− TCRβlo and CD4−CD8+ TCRβhi cells in L-DKO mice are mostly iNKT cells and type II NKT cells.

iNKT cells are known to express lower levels of TCR than those of conventional CD8+ T cells and are phenotypically classified into either the CD4+CD8− or CD4−CD8+ compartment. The development of iNKT cells also begins in the neonatal stage (4) with a time frame similar to the CD4+CD8− TCRβlo cells and CD4−CD8+ TCRβhi cells in L-DKO mice. We therefore examined the possibility that the TCRβhi cells in L-DKO mice were iNKT cells. FACS analysis of thymocytes revealed that most CD4+ cells and a third of CD4−CD8− cells in L-DKO mice were recognized by an Ag-loaded CD1d tetramer that specifically binds to the canonical TCR on iNKT cells (Fig. 3A). PLZF has been shown to be a signature transcription factor for innate T lymphocytes, including Vγ1Vδ6.3 γδ T cells and iNKT cells (35). Intracellular staining with anti-PLZF Ab demonstrated an overall increased in expression of PLZF in CD4+CD8− and CD4−CD8+ cells but not DP cells in L-DKO mice (Fig. 3B). PLZF expression was found in both αβ+ and γδ+ fractions of CD4−CD8− cells in the mutant mice. Furthermore, a small fraction of TCR αβ+CD8− cells also expressed PLZF, indicating their possible lineage relationship with TCRγδ PLZF-expressing cells. On average, the absolute number of CD4+DP and DN fractions in L-DKO mice were −9- and 28-fold higher, respectively, than those of the age-matched Cre− controls (Fig. 3C). Because most CD4+ DP and DN fractions in L-DKO mice express PLZF, we conclude that Id2 and Id3 double deletion leads to a significant increase in numbers of PLZF-expressing thymocytes. Sequencing analysis of cDNA products amplified with Vα14- and Jα18-specific primer indicated the exclusive usage of the canonical invariant Vα14Jα18 TCR in CD4 CD1d tetramer− isolated from L-DKO mice (Fig. 3D).

The lipid Ag used in the CD1d tetramer specifically recognizes iNKT cells. Type II NKT cells have been shown to use several highly restricted VaJα rearrangements, including Va3 and Jα9 rearrangement (7, 8). Therefore, we examined Va3Jα9 usage in the TCRβ14CD1d tetramer− CD4−CD8− and CD4+CD8− cells in the mutant mice. Va3Jα9 products but not Va14Jα18 products were readily detected by PCR in TCRβ14CD1d tetramer− cells (Fig. 3E). Sequence analysis of Va3Jα9 products from TCRβ14CD1d tetramer− cells indicated that a third of them were in-frame and the remaining two-thirds were out-of-frame Va3Jα9 rearrangements (Fig. 3F). Therefore, other Va rearrangements must also be involved in generating functional TCRα-chains in the TCRβ14CD1d tetramer− fraction.

To further test lineage identity of the expanded TCRβlo cells in L-DKO mice, we bred L-DKO mice to the MHC class II-deficient background. Both TCRβ14CD1d tetramer− and TCRβ6CD1d tetramer− cells in L-DKO mice were generated in the absence of MHC class II selection (Fig. 4), demonstrating that these cells were unrelated to the conventional helper T cell lineages. Thus, we conclude that Lck-Cre–mediated deletion of Id2 and Id3 resulted in increased generation of both iNKT cells and type II NKT cells.

Expansion of stage 1 iNKT cells in L-DKO mice

Further analysis showed that most iNKT cells developed in L-DKO mice do not express NK1.1 and DX5 even though they have downregulated CD24 (Fig. 5A). FACS analysis also showed that iNKT cells in L-DKO mice expressed low levels of CD44 (Fig. 1A). These features together with PLZF expression indicated that iNKT cell development in L-DKO mice has progressed to stage 1 (36, 37), where cells undergo proliferative expansion (38). Indeed, in vivo BrdU pulse labeling revealed a significantly higher fraction of cycling cells among CD4+ population in L-DKO mice (most of which are iNKT cells) in comparison with the conventional CD4 SP cells in Cre− controls (Fig. 5B, 5C). The percentage of cycling cells was also higher in the DN TCRβlo fraction of L-DKO mice (most of which are presumed type II NKT cells) in comparison with the conventional CD4 SP cells in Cre− controls. The same analysis indicated a relative normal pattern of cell cycle for L-DKO mice at the DN2, DN3, and DP stages of T cell development (Fig. 5C, Supplemental Fig. 3). This proliferative behavior of NKT cells was observed at both weaning and young adult age (Fig. 5B). Repertoire analysis with a panel of TCR Vβ isotype-specific Abs revealed a broad pattern of Vβ usage among CD4+ cells of L-DKO mice, indicating that the expanded iNKT cells in L-DKO mice remain polyclonal (Table I). However, the overall patterns of Vβ usage in L-DKO mice were different from the conventional Vβ8 > Vβ7 > Vβ2 rule (39), indicating the possibility of an altered selection during development or expansion phase of these iNKT cells. Furthermore, five of six L-DKO mice showed increased Vβ usage involving Vβ8.3, Vβ11, or Vβ13. Taken together, these results indicated that Id2 and Id3 deletion resulted in an expansion of immature iNKT cells.

Id2 and Id3 deletion results in a biased rearrangement toward Va14-Jα18

Given that iNKT cell development is dependent on Va14 to Jα18, which typically occurs during the DP window of thymocyte development, we asked whether Id2 and Id3 deletion affects TCRα rearrangement. We assessed the Jα usage by performing high-throughput sequencing of rearrangement products involving either Vα8 or Vα14 genes from the preselecting CD4+CD8−/CD69− DP cells. CD1d tetramer− and CD69+ postselecting cells were gated out to avoid the contamination of NKT cells or mature αβ T cells (Supplemental Fig. 4). Jα usage was captured by PCR amplification with a Va-specific primer and a Cα primer. A broad pattern of Jα usage involving Vα8 rearrangements was detected in both Cre− control and L-DKO cells (Fig. 6A). Because Vα8 is one of the commonly used Vα genes (40), the random distribution of Jα usage indicates that a significant fraction of DP cells undergoes relatively normal TCRα rearrangements. In contrast to the Vα8 result, Vα14 rearrangements exhibited a highly skewed pattern toward Jα18 usage in L-DKO mice (Fig. 6B). This bias toward Jα18 was associated with Vα14 but not Vα8 (Fig. 6A). It remains a possibility that some of these Vα14-Jα18 rearrangements may come from NKT cells that have downregulated their surface TCR. However, this argument cannot fully explain the fact that a significant number of unproductive Vα14-Jα18 rearrangements were also observed in three independent L-DKO DP samples (18, 19, and 61%; Fig. 6B, lower panel). In particular, one of the three samples (Fig. 6B, center pie chart) showed more unproductive rearrangements (61%) than productive rearrangements (37%), which presents a typical preselecting repertoire. In contrast, unproductive Vα14-Jα18 rearrangements were detected at a much lower frequency in three wild-type control samples (0, 0.01, and 5%; Fig. 6B, upper panel). This result suggests that Id2 and Id3 deletion promotes Vα14 to Jα18 rearrangements among developing T cells when Vα14 is involved in rearrangements.

Increased iNKT development in Id2- and Id3-deficient mice is driven by high levels of E proteins

The major targets of Id proteins are E protein transcription factors, although E proteins are not the only Id-interacting proteins reported thus far (41). To test whether enhanced iNKT development is indeed regulated by E proteins, we lowered the E protein dosage by removing one copy each of the E2A and HEB genes on
FIGURE 3. CD4+CD8-TCR<sup>b</sup> and CD4+CD8-TCR<sup>d</sup> cells in L-DKO mice are NKT cells. (A) Representative staining of total lymphocytes with CD4, CD8, TCR<sub>b</sub>, and CD1d<sup>tet</sup>. CD4 and CD8 staining of total thymocytes were used to define DN and CD4 SP gates. DN and CD4 fractions of each genotype were analyzed with either unloaded CD1d<sup>tet</sup> as a control or PBS57-loaded CD1d<sup>tet</sup>. The percentages of CD1d<sup>tet</sup>+ or TCR<sub>b</sub>+CD1d<sup>tet</sup>-cells in CD4 and DN fractions are displayed in the plots. (B) Intracellular staining of PLZF in CD4 SP (CD4+CD8<sup>-</sup>), DP (CD4<sup>-</sup>CD8<sup>-</sup>), and DN (CD4<sup>-</sup>CD8<sup>-</sup>) fraction of total lymphocytes. DN fraction is further separated by TCR<sub>b</sub> and TCR<sub>d</sub>. DN TCR<sub>b</sub>lo, DN TCR<sub>d</sub>lo, and DN TCR<sub>d</sub>+ populations were analyzed. (C) Cell counts for CD1d<sup>tet</sup> iNKT cells in CD4 SP and DN fractions in 20-d-old pups. The mean values for CD4 SP iNKT cells are 1.43 ± 0.31 x 10<sup>5</sup> and 12.76 ± 2.34 x 10<sup>5</sup> in Cre<sup>-</sup> controls and L-DKO, respectively, with <i>p</i> = 0.0013. The mean values for DN iNKT cells are 0.64 ± 0.15 x 10<sup>5</sup> and 17.81 ± 5.08 x 10<sup>5</sup> in Cre<sup>-</sup> controls and L-DKO, respectively, with **<i>p</i> = 0.0097. Five mice of each genotype group were used in the analysis. (D) Sequence results of V<sub>α</sub>14J<sub>α</sub>18 junctions from cDNA of DN CD1d<sup>tet</sup>+ and CD4 CD1d<sup>tet</sup>- populations in L-DKO mice. N additions are shown in (Figure legend continues)
the L-DKO background (referred to as L-DKO50%E). This genetic change effectively reduced iNKT cell numbers almost back to the level seen in Cre2 controls and concurrently enhanced numbers of gd T cells dramatically (Fig. 7A, 7B). Further analysis confirmed that the expanded gd T cells in L-DKO50%E mice belong to the innate gd T lineage that uses exclusively the Vd6.3 TCR (Fig. 7A, right panel). This result indicates that innate gd T and iNKT lineages are regulated by different levels of E proteins.

To further evaluate the necessity of E proteins in the generation of innate gd T cells, we generated Id2f/fId3f/fE2Af/fHEBf/fLck-Cre+ quadruple-deficient mice (referred to as L-QKO mice). Deletion of E2A and HEB effectively prevented ab T and iNKT lineage development (Fig. 7C). Numbers of γδ T cells in L-QKO mice were also reduced to 10% of the Cre2 controls (Fig. 7D), a phenotype similar to the previously defined Lck-Cre–mediated E2A and HEB knockout mice (19). This result demonstrated that the Id2 and Id3 genes control iNKT lineage development through inhibition of E protein activities exclusively.

**Discussion**

Following a recent publication demonstrating an essential role for Id3 and Id2 at the TCR selection checkpoint (22), the present study provided new genetic evidence that Id2 and Id3 are also collectively involved in regulating the pre-TCR checkpoint. Furthermore, our

**FIGURE 4.** Development and expansion of iNKT cells in L-DKO mice is independent of MHC class II. Total thymocytes were analyzed with either CD4 and CD8a (first row across) or CD1dtet and TCRγδ (second row across) staining. The CD4+CD8+ (third row across) and CD4+CD8− (fourth row across) fractions of thymocytes were displayed by CD1dtet and TCRβ expression. Samples are from 20-d-old Id2f/fId3f/fLckCre−, Id2f/fId3f/fLckCre+, Id2f/fId3f/fLckCre− MHC class II+/−, and Id2f/fId3f/fLckCre−MHC class II−/− mice as indicated on the top of each column. Percentages of cells in each quadrant are displayed. n = 2 for each genotype.

bold. (E) PCR analysis of Vα14Jα18 and Vα3Jα9 rearrangement in CD1dtet+ and CD1dtet− fraction of DN and CD4 cells. Results are representative of two independent experiments involving separate L-DKO mice. (F) Sequence results of Vα3Jα9 junctions from cDNA of TCRβloCD1dtet2 population in L-DKO mice. N additions are shown in bold.
FIGURE 5. Characterization of NKT cells in L-DKO mice. (A) Total thymocytes from Cre− control L-DKO mice were displayed for CD1d tet and CD24 expression (left column), NK1.1 and TCRβ expression (middle column), and DX5 and TCRβ expression (right column). (B) BrdU incorporation among DP and CD4+ SP thymocytes analyzed at 4 h after BrdU injection of 20-d-old (left panel) or 2-mo-old mice (right panel). The percentage of BrdU+ cells for each subpopulation is shown in histograms. (C) Summary of BrdU+ percentages in DN2, DN3, DP, CD4+CD8−, and DN TCRβ+ fractions from three independent experiments of 20-d-old pups. Significant difference was observed between L-DKO and control mice among the CD4+CD8− fraction (mean value, 3.85 ± 0.61 and 8.56 ± 0.72% for Cre− controls and L-DKO, respectively, with **p = 0.0076). Numbers of DN TCR+ cells in the Cre− control group were too small to be included in this analysis.
study also revealed a previously unanticipated role for \textit{Id3} and \textit{Id2} in regulating iNKT cell development.

Two models have been proposed to explain the development of iNKT cells (13). The “pre-committment” model postulates that iNKT cell fate is predetermined prior to CD1d-mediated selection. This idea has been supported by the finding that \textit{V\textalpha 14-\textit{Ja18}} rearrangements can be detected prior to the appearance of conventional \textit{\alpha\beta} T cells in the mouse fetus (12). However, this finding is inconsistent with the fact that most \textit{V\textalpha 14-\textit{Ja18}} rearrangements occur as secondary rearrangements in DP cells, from which iNKT cells are generated continuously in postnatal life (9–11). The “mainstream” (or TCR- instructive) model argues that iNKT cells acquire their lineage identity upon CD1d-mediated TCR selection of DP cells that have successfully produced the canonical \textit{V\textalpha 14-Ja18} TCR resulting from the sequential rearrangement of the TCR\textalpha gene segments. Recent studies further demonstrated that a strong TCR signal is associated with activation of NKT lineage-specific transcription factors such as PLZF (42). Mounting evidence supports the idea that CD1d-mediated selection, together with signaling events involving the SLAM receptors, drive iNKT lineage differentiation (37). In light of these previous findings, our observation of a biased \textit{V\textalpha 14-\textit{Ja18}} rearrangement in L-DKO mice provides an alternative view to the existing models. We propose that \textit{V\textalpha 14-Ja18} rearrangement is not a random event and is subject to repression by concerted activity of \textit{Id3} and \textit{Id2}.

Multiple factors may contribute to the overall increase in numbers of iNKT cells in L-DKO mice. The \textit{Ja} repertoire analysis of L-DKO DP cells clearly revealed a biased usage of \textit{V\textalpha 14-\textit{Ja18}} when \textit{V\textalpha 8} is used in rearrangement. Because \textit{Ja18} usage was not altered when \textit{V\textalpha 8} was used in rearrangements, the biased \textit{V\textalpha 14-Ja18} rearrangement in D-LKO mice cannot be simply due to targeted regulation at the \textit{Ja18} site. Given that each \textit{Ja} gene is regulated by an independent promoter, we propose that \textit{V\textalpha 14} may be subject to targeted regulation in L-DKO mice. However, \textit{V\textalpha 14} must be working in concert with \textit{Ja18} in L-DKO mice to promote \textit{V\textalpha 14-Ja18} usage. This biased \textit{V\textalpha 14-Ja18} usage may only affect a small fraction of DP cells because the pattern of \textit{V\textalpha 8} rearrangements, a relatively common \textit{V\textalpha 8}, seems unperturbed in L-DKO mice. Therefore, altered \textit{V\textalpha 14-Ja18} usage alone is not sufficient to explain the overall increase in iNKT cell numbers. Our study has further revealed that most iNKT cells detected in the thymus of L-DKO mice are proliferating immature iNKT cells. The expansion of immature iNKT cells after CD1d rearrangement could be another reason for the increase of iNKT cells in L-DKO mice.

The expansion of iNKT cells in L-DKO mice could also be attributed to other T lineage cells developing along with iNKT cells that may inadvertently affect the development and expansion of iNKT cells (43). These include the innate $\gamma\delta$ T cells that developed in the neonatal stage and the small number of conventional CD4 SP cells made through positive selection (22). Indeed, CD4 SP cells that developed in Id3-deficient background have been shown to exhibit various effector phenotypes (44), which could potentially influence the development and expansion of iNKT cells. To resolve this issue, we have tested our L-DKO mice on an MHC class II-deficient background and observed a similar expansion of iNKT cells as in L-DKO mice. The effect of innate $\gamma\delta$ T cell on the development of iNKT cells in our L-DKO model could be further investigated in the future by crossing the L-DKO mice to the TCR8-deficient background.

Under our experimental conditions, the innate $\gamma\delta$ lineage and iNKT lineage are selectively expanded in response to increasing levels of E proteins. The tight correlation between E protein dosage and unique TCR types such as $V\gamma 1.1V\delta 6.3$ of innate $\gamma\delta$ T cells and $V\alpha 14J\alpha 18$ of iNKT cells provides a genetic frame-
work for further understanding how TCR rearrangement, expression, and signaling are coupled with E protein-mediated lineage differentiation programs.

Investigation of PLZF expression shed new light on the lineage relationship between \( V_{\gamma 1.1} V_{\delta} \) \( \gamma \delta \) T cells and iNKT cells (45, 46). Although these two innate lineages seem to be developed independently during thymopoiesis, they clearly share a similar developmental blueprint by employing PLZF-mediated transcriptional regulation and possess similar innate-like features such as restricted TCR usage, acquisition of effector memory like phenotypes upon maturation, and coexpression of IL-4 and IFN-\( \gamma \) (35). Furthermore, a recent RNA expression profiling analysis classified iNKT cell as a lineage closely related to \( \gamma \delta \) T cells (47). This result supports the idea that NKT cells could be evolutionarily
FIGURE 7. E protein dosages at the DN3 stage control lineage outcomes. (A) Effects of E protein dosage on T cell development revealed by analysis of Cre control, L-DKO, and L-DKO50%E (Id2^{+/+}Id3^{+/+}E2A^{+/+}HEB^{+/+}Lck-Cre') mice. Total thymocytes were analyzed with either CD4 and CD8 staining (far left column), CD1dTet and TCRβ staining (middle left column), or CD1dTet and TCRδ staining (middle right column). TCRδ+ cells were further analyzed for Vδ6.3 expression (far right column). (B) Cell counts of each T cell fractions as defined in (A). Three mice for each genotype group were included in the analysis. (C) FACS analysis of L-QKO mice. Results of CD4 and CD8 analysis of total thymocytes (left column) are shown along with the CD1dTet and TCRβ analysis of gated DN fractions (right column). Results are representative of three pairs of animals. (D) Cell counts for total thymocytes (top) and γδ T cells (bottom) in the thymus of 20-d-old L-QKO mice. n = 3 pairs, p < 0.0005 for both plots.
closer to innate γδ T cells than the conventional T cells that perform adaptive immune functions. Our study raised the possibility that E protein–mediated regulation may function upstream of PLZF and other innate lineage-relevant transcription factors. The genetic models established in this study identified E proteins as an important transcriptional switch controlling lineage choice between nNK T and other alternative T cell lineages.

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Disclosures

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