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Id3 and Id2 act as a dual safety mechanism in regulating the development and population size of innate-like γδ T cells

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Abstract

The innate-like T cells expressing Vγ1.1 and Vδ6.3 represent a unique T cell lineage sharing features with both the γδ T and the invariant NKT cells. The population size of $V\gamma1.1^+V\delta6.3^+$ T cells is tightly controlled and usually contributes to a very small proportion of thymic output, but the underlying mechanism remains enigmatic. Deletion of *Id3*, an inhibitor of E-protein transcription factors, can induce an expansion of the $V\gamma1.1^+V\delta6.3^+$ T cell population. This phenotype is much stronger on the C57Bl/6 background than on the 129/sv background. Using quantitative trait linkage analysis, we identified *Id2*, a homologue of *Id3*, to be the major modifier of *Id3* in limiting $V\gamma1.1^+V\delta6.3^+$ T cell expansion. The $V\gamma1.1^+V\delta6.3^+$ phenotype is attributed to an intrinsic weakness of *Id2* transcription from *Id2* C57Bl/6 allele, leading to an overall reduced dosage of Id proteins. However, complete removal of both *Id2* and *Id3* genes in developing T cells suppressed the expansion of $V\gamma1.1^+V\delta6.3^+$ T cells due to decreased proliferation and increased cell death. We showed that conditional knockout of *Id2* alone is sufficient to promote a moderate expansion of γδ T cells. These regulatory effects of *Id2* and *Id3* on $V\gamma1.1^+V\delta6.3^+$ T cells are mediated by titration of E protein activity, since removing one or more copies of E protein genes can restore $V\gamma1.1^+V\delta6.3^+$ T cell expansion in *Id2* and *Id3* double conditional knockout mice. Our data indicated that *Id2* and *Id3* collaboratively control survival and expansion of the γδ lineage through modulating a proper threshold of E-proteins.

Keywords

Id3; Id2; γδ T cells; E-proteins; PLZF; modifier mapping

Introduction

γδ T cells are a subset of T lymphocytes generated in the thymus that function between the innate and adaptive immune system. They have features of the adaptive immune system, such as the expression of variable rearranged γδ T cell receptors, but they also have features of the innate immune system, such as the ability to respond to stimulation rapidly (1). They can directly lyse infected or stressed cells as well as interact with αβ T cells, B cells and dendritic cells and regulate their functions (2). As a result, γδ T cells are involved in a broad range of immune processes, such as infection, inflammation, autoimmunity, tumor surveillance and tissue maintenance (1, 3). These cells are produced in large numbers in the fetal and neonatal stages in mammals, disseminating and forming stable populations in

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Conflict of Interest
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internal organs, mucosal and body surfaces, but their thymic production is gradually replaced by αβ T cells when the animal matures (4). The mechanism that controls the developmental switch from γδ to αβ T cell production in the thymus is not fully understood.

Among γδ T cells, the cells that express the Vγ1.1 and Vδ6.3 segments of the γδ T cell receptor belong to a unique subset. In mice, these cells are produced in large numbers in the neonatal thymus (5) and are capable of rapidly producing multiple cytokines, including IFNγ and IL-4, upon stimulation (6). They express the transcription factor PLZF that is also found in NKT cells (7). Like NKT cells, they also have a significant presence in the liver (8). The semi-invariant nature of their T cell receptor and their response pattern lead to the classification of these cells as "innate-like γδ T cells." Although their function is not clearly understood, several studies pointed out that these cells may play an important role in attenuating excessive inflammation during infection and autoimmune processes due to their unique ability among γδ T cells to produce Th2-like cytokines (as reviewed by Carding SR. and Egan PJ. (3)). The population size of $V\gamma1.1^+V\delta6.3^+$ γδ T cells varies between mouse strains; they are particularly abundant in the DBA strain (in which usually a Vδ6.4 segment is expressed) but relatively rare in the B6 strain (6). However, in the absence of a helix-loop-helix transcription regulator, *Id3*, it has been shown that $V\gamma1.1^+V\delta6.3^+$ γδ T cells can also expand dramatically in mice with B6 genetic background (9).

Id3 has been implicated to play both positive and negative roles in the developmental control of γδ T cells. It has been shown that in developing DN thymocytes, if a cell successfully rearranges the γδ T cell receptor genes, the surface expression of γδ T cell receptor can send a strong signal into the cell and up-regulate *Id3*, promoting the cell to adopt a γδ T cell fate (10). However, *Id3* also plays a distinct inhibitory role controlling the development of $V\gamma1.1^+V\delta6.3^+$ γδ T cells because this population is dramatically expanded in *Id3* deficient mice. More interestingly, this expansion is limited to the neonatal window and cannot be recapitulated by transferring *Id3*-deficient bone marrow cells into adult wild type B6 animals (11). The expansion also requires a pure B6 genetic background; in a B6/129 mix background, the expansion is variable and often greatly diminished (9). The latter phenomenon suggests that additional gene(s) specific to the B6 background is also critical in the development of $V\gamma1.1^+V\delta6.3^+$ γδ T cells in the absence of *Id3*.

While the importance of *Id3* in regulating the development and population size of γδ T cells has been firmly established, the underlying mechanism is still poorly defined. This strain- and genotype-specific expansion of $V\gamma1.1^+V\delta6.3^+$ γδ T cells represents a unique opportunity to identify novel players in the developmental control of γδ T cells. We designed a backcross experiment between B6 and 129 *Id3*-deficient mice with a goal to identify the background genes in regulating the $V\gamma1.1^+V\delta6.3^+$ γδ T cells. We found that another member of the Id protein family, *Id2*, was the major modifier of *Id3* involved in the control of γδ T cell population size. *Id2* 129 allele is expressed more in γδ T cells than *Id2* B6 allele; it is highly expressed in $V\gamma1.1^+V\delta6.3^+$ γδ T cells and mature γδ T cells in general. Conditional knockout of *Id2* leads to expansion of γδ T cells not limited to the $V\gamma1.1^+V\delta6.3^+$ subset. Paradoxically, if both *Id2* and *Id3* are completely deleted, the $V\gamma1.1^+V\delta6.3^+$ γδ T cells actually fail to accumulate, possibly due to attenuated proliferation and increased cell death induced by unrestricted E protein activity. We further showed that these phenomena may occur after γδ T cell lineage commitment, thus separating them from the role *Id3* plays in the initial TCR signaling and lineage choice processes. These results clearly demonstrated the interweaving roles of Id proteins and E proteins in the control of γδ T cell development.

Materials and Methods

Mice

The Id3$^{-/-}$ (12), Id2^{GFP} (13), Id2$^{f/f}$ (14), Id3$^{f/f}$ (15), E2A$^{f/f}$ (16), HEB$^{f/f}$ (17) and LckCre transgenic (18) mice have been described previously and all maintained on pure B6 background. C57BL/6J, 129X1/SvJ mice were purchased from The Jackson Laboratory. CD4Cre transgenic mice on B6 background were purchased from Taconic. Animals were bred and maintained in the SPF facility managed by Duke University Division of Laboratory Animal Research. All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Flow cytometry

The antibodies used in the flow cytometry analyses were as follows: anti-mouse CD4 (GK1.5), anti-mouse CD8a (53-6.7), anti-mouse B220 (RA2-6B2), anti-mouse/human CD44 (IM7), anti-mouse CD25 (3C7), anti-mouse NK-1.1(PK136), anti-mouse Ly-6G/Ly-6C(Gr-1) (RB6-8C5), anti-mouse CD11b(M1/70), anti-mouse TCRγ/δ(GL3), anti-mouse TCR Vγ1.1 (2.11), anti-mouse CD24 (M1/69) and anti-mouse TCRβ (H57-597) were purchased from Biolegend. The PE anti-mouse Vδ 6.3/2 (8F4H7B7) antibody, annexin V and the APC BrdU Flow Kit were purchased from BD Biosciences. 7-Aminoactinomycin D (7-AAD) was purchased from Life Technologies.

Single-cell suspensions were prepared from thymus, spleen and peripheral lymph nodes, and suspended in cold FACS buffer (1×PBS supplemented with 5% bovine calf serum). 1×10^6 cells were stained with antibodies in the dark at 4°C for 30 min. After washing with cold FACS buffer, cell suspensions were analyzed on a FACSCanto II flow cytometer (BD Biosciences). FlowJo software (Tree Star) was used for data analysis. Cell sorting was performed with a FACS DiVa sorter (BD Biosciences).

Quantitative trait linkage analysis

Id3$^{-/-}$ mice on B6 background were crossed with 129X1/SvJ mice to generate Id3$^{+/-}$ F1 mice. F1 mice were backcrossed with Id3$^{-/-}$ mice on B6 background to generate Id3$^{-/-}$ F2 mice. The genomic DNA was extracted from toes of Id3$^{-/-}$ F2 mice and sent to Genomic Analysis Facility at Duke University for single nucleotide polymorphism (SNP) analysis using a 377 genome-wide mouse SNP panel (Illumina). Genome-wide scans were plotted using J/QTL mapping program (version 1.3) (http://churchill.jax.org/software/jqtl.shtml), and genomic regions with significant linkage to the expansion of Vγ1.1$^+$ Vδ6.3$^+$ γδ T cells (>1% of total thymocyte) was determined using methods previously described (19). Additional DNA primers were designed to PCR-amplify regions on chromosome 12 near the centromere end, and the PCR products were sequenced to determine the status of additional SNP markers around this region.

Real-time PCR analysis

TCRγδ$^+$ Vδ 6.3$^+$ cells were sorted from mouse thymus, and total RNA was extracted with RNAqueous micro kit (Life Technologies). Reverse transcription was performed with M-MLV reverse transcriptase (Life Technologies). SYBR-based real-time PCR was performed to quantitatively compare gene expression, normalized to β-actin. QPCR primer sequences are available upon requests.

Restriction fragment length polymorphism analysis

A 481bp fragment was PCR amplified from *Id2* cDNA made from thymic TCRγδ$^+$ Vδ 6.3$^+$ cells. The PCR product was digested with EcoRI. The 481bp PCR product from B6 *Id2*

allele does not contain any EcoRI restriction site. The product from 129 *Id2* allele contains one EcoRI site, and digestion with the enzyme will generate one 393bp fragment and one 88bp fragment.

In vitro stimulation of γδ T cells

Thymic GFP-negative TCRγδ$^+$ cells from Id2$^{GFP/+}$ mice were sorted and cultured in OP9-DL1 cell covered wells with MEM-α medium, supplemented with 10% fetal bovine serum and 5 ng/mL IL-7, with or without 1 μg/mL anti-TCRγδ (clone UC7-13D5, Biolegend). Cells were harvested after 5 days for FACS analysis.

In vivo BrdU incorporation assays

1 mg of BrdU was i.p. injected to each mouse 15 hours (for Id2$^{f/f}$ CD4Cre$^+$ vs. Id2$^{f/f}$ CD4Cre$^-$ mice experiments) or 4 hours (for *Id2* and *Id3* double conditional knockout experiments) before sacrificing the mice. Cells were harvested and processed with the BrdU Flow Kit (BD Biosciences) according to manufacturer protocol.

Cell death analysis

For Id2$^{f/f}$ CD4Cre$^+$ vs. Id2$^{f/f}$ CD4Cre$^-$ mice experiments, thymocytes were harvested and directly analyzed with 7AAD/annexin V for cell death according to manufacturer protocol (BD Biosciences). For Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ vs. Id2$^{f/B}$ Id3$^{f/f}$ CD4Cre$^+$ mice experiments, TCRγδ$^+$ Vδ 6.3$^+$ cells were first sorted from mouse thymus and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 55μM of 2-mercaptoenthanol for 24 hours prior to 7AAD/annexin V analysis.

Statistical analysis

Sample data was compared using Student's t test, and p value less than 0.05 was considered significant.

Results

Backcross mapping identifies a single locus that modulates numbers of Vδ6.3$^+$ cells in *Id3* knockout mice

We analyzed the thymus of *Id3* deficient mice on B6 or B6/129 mix background and showed that the population of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells is consistently large in mice with B6 background but variable in mice with B6/129 mix background **(Fig 1A, S1A)**. Because Vδ6.3 usage is uniquely associated with the Vγ1.1$^+$Vδ6.3$^+$ population found in *Id3* deficient mice(11), Vδ6.3 was used as a lineage marker in subsequent genetic analysis. We hypothesized that an *Id3*-modifying gene(s) is responsible for the phenotypic difference observed between these two strains. We designed a backcrossing strategy to further verify our hypothesis **(Fig 1B)**. First, Id3$^{-/-}$ mice on B6 background were crossed with wild type 129X1/SvJ mice. The resulting F1 mice (Id3$^{+/-}$ with mixed background) were further backcrossed with Id3$^{-/-}$ mice on B6 background. Half of the F2 progeny from the backcross were expected to be Id3$^{-/-}$, which were analyzed for their Vδ6.3$^+$ γδ T cell percentage in the thymus. Among all the 226 Id3$^{-/-}$ F2 mice analyzed, we found a wide range of distribution in Vδ6.3$^+$ γδ T cell percentage **(Fig 1C)**. As a comparison, most Id3$^{-/-}$ mice on B6 background have >1% Vδ6.3$^+$ γδ T cell among thymocytes **(Fig 1C)**. This result suggests the existence of one or more possible *Id3* modifier gene, with the 129 allele being dominant (inhibit Vδ6.3$^+$ γδ T cell expansion), and the B6 allele being recessive (permit Vδ6.3$^+$ γδ T cell expansion). In order to identify the potential gene(s), we performed genome-wide SNP analysis in 25 Id3$^{-/-}$ F2 mice. We found that B6 homozygosity of a single 30 Mb region on chromosome 12 near the centromere was strongly linked with the presence of high numbers

(>1%) of Vδ6.3$^+$ γδ T cells (LOD score >2) **(Fig 1D)**. Focusing on one SNP on chromosome 12 location 30Mb, we analyzed additional 138 Id3$^{-/-}$ F2 mice and showed that B6/B6 homozygosity of this location was significantly correlated with the accumulation of Vδ6.3$^+$ γδ T cells **(Fig. S1B)**. We further analyzed these 138 Id3$^{-/-}$ F2 mice, determining their genotype of two SNP markers on chromosome 12 (location around 3Mb and 30Mb) flanking the region identified in the genome-wide SNP analysis. We looked for mice showing discordant genotype at these two loci (one being B6/B6, the other being B6/129) and with more than 1% Vδ6.3$^+$ γδ T cells in their thymi. We then performed detailed study of additional SNP markers inside this region and narrowed down the critical interval to a 3 Mb region (chromosome 12 location 25.16Mb-28.02Mb) **(Fig 1E)**. There are only four known protein coding genes in this interval, including *Sox11, Cmpk2, Rsad2, and Rnf144a* **(Fig 1F)**. The other features (*SNORA17, AC155270.1, 5s_rRNA*) are either RNA coding genes or putative genes. *Id2*, a homolog of *Id3*, locates immediately outside of the boundary (25.09Mb), leaving the possibility that its expression may still be influenced by regulatory elements inside this interval.

Id2 is a major modifier of *Id3* in regulating the population size of Vδ6.3$^+$ cells

To determine which gene in this region on chromosome 12 is truly responsible for the accumulation of Vδ6.3$^+$ γδ T cells in Id3$^{-/-}$ mice with B6 background, we first examined allelic variations between 129 and B6 mice in terms of their protein-coding sequences. We did not find non-synonymous changes or splice site SNPs for *Sox11, Cmpk2, Rnf144a* and *Id2* genes. Several non-synonymous changes were identified in the *Rsad2* gene. However, transgenic rescue tests showed that the 129 allele of *Rsad2* cannot prevent Vδ6.3$^+$ γδ T cells from accumulating when introduced into conditional *Id3* knockout mice on B6 background **(Fig S2 A, B)**.

We considered the alternative possibility that variation in non-protein coding sequences between 129 and B6 mice may affect the expression level of one of these candidate genes. We compared the mRNA expression level of all the aforementioned candidate genes in thymic Vδ6.3$^+$ γδ T cells from Id3$^{-/-}$ mice with either pure B6 or B6/129 F2 mix background on chromosome 12 position 25.16Mb-28.02Mb. We found that only *Id2* is expressed differently between the groups, being more highly expressed in mice with B6/129 F2 mixed background **(Fig 2A and Fig S2C)**. In order to verify that the increased expression comes from the 129 *Id2* allele, we performed restriction fragment length polymorphism analysis of the *Id2* cDNA made from the γδ T cells. The 129 *Id2* allele contains an EcoRI restriction site in the 3' UTR which is absent in the B6 allele. We amplified the *Id2* cDNA region surrounding the EcoRI site by PCR and digested the product with EcoRI, and we observed abundant restriction fragments generated from the 129 allele **(Fig 2B)**. These results indicate that a potential regulatory element on chromosome 12, between position 25.16Mb-28.02Mb, acts *in cis* to control *Id2* expression. The 129 version of the element may induce a higher level of *Id2* expression than the B6 version, and this higher *Id2* level may inhibit the expansion of Vδ6.3$^+$ γδ T cells in a dominant manner.

In order to further examine this hypothesis, we took advantage of the Id2^f allele (as compared to Id2^B, indicating the wild type allele in B6 background). This floxed allele was generated with mouse embryonic stem cells from the 129 strain (14). Although this allele has been backcrossed to the B6 genetic background for more than 10 generations, the region on chromosome 12 around *Id2*, including location 25.16Mb-28.02Mb, remained of 129 origin. In the absence of Cre, this allele behaves similarly to the wild type allele from the 129 strain. By introducing this allele into the Id3$^{-/-}$ mouse on B6 background, we can specifically investigate the role of this region on the development of Vδ6.3$^+$ γδ T cells. We found that introducing one copy of the Id2^f allele was sufficient to significantly suppress the

accumulation of Vδ6.3$^+$ γδ T cells **(Fig 2C)**. In order to determine whether it is *Id2* itself that is limiting the development of Vδ6.3$^+$ γδ T cells, we knocked out *Id2* and *Id3* in T cells specifically with CD4Cre. The Id2$^{B/B}$ Id3$^{f/f}$ CD4Cre$^+$ mice behaved similarly to Id3$^{-/-}$ mice, showing significant accumulation of Vδ6.3$^+$ γδ T cells **(Fig 2D)**. However, unlike the Id2$^{f/B}$ Id3$^{-/-}$ mice, the 129 genetic material in the Id2$^{f/B}$ Id3$^{f/f}$ CD4Cre$^+$ mice could not suppress the accumulation of Vδ6.3$^+$ γδ T cells, demonstrating that it is indeed *Id2* that is playing the inhibitory role **(Fig 2D)**. These results indicate that genetic material on chromosome 12 location 25.16Mb-28.02Mb from the 129 background may influence the expression of *Id2*, thus inhibiting the accumulation of Vδ6.3$^+$ γδ T cells together with *Id3*.

We hypothesized that double deletion of *Id2* and *Id3* with CD4Cre might lead to further increase of the Vδ6.3$^+$ γδ T cells. To our surprise, Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ mice actually have very few of these cells **(Fig 2D, E)**. Because the Vδ6.3$^+$ γδ T cells proliferate vigorously during the neonatal stage, we examined thymocytes from 7 days old mice and found that, regardless of the *Id2* genotype, Vδ6.3$^+$ γδ T cells from Id3$^{f/f}$ CD4Cre$^+$ mice were more highly proliferative than those from the Cre$^-$ controls, but the proliferation is slightly attenuated in Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ mice **(Fig 3A)**. When we cultured the cells *in vitro* for 24 hours, we found that cells from Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ mice showed more rapid cell death than those from Id2$^{f/B}$ Id3$^{f/f}$ CD4Cre$^+$ mice **(Fig 3B)**. We performed QPCR for a panel of cell-death-related genes and found that two proapoptotic genes, *Bax* and *Bim*, were significantly up-regulated in Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ cells **(Fig 3C)**. These results indicate that complete removal of both Id2 and Id3 proteins can actually be inhibitory for Vδ6.3$^+$ γδ T cell survival and proliferation, implying that the maximal output of Vδ6.3$^+$ γδ T cells requires an optimal level of Id proteins.

Id2 functions as an inhibitor of γδ T cell development

Since complete knockout of both *Id2* and *Id3* is detrimental to the accumulation of Vδ6.3$^+$ γδ T cells, one possible explanation for the expansion of these cells in Id2$^{B/B}$ Id3$^{-/-}$ mice on B6 background is that the lower expression level from the B6 version of *Id2* allele results in a higher, but not too high, activity of E proteins to drive Vδ6.3$^+$ γδ T cell expansion. If so, removing *Id2* alone may also result in an expansion of γδ T cells -- at least in some subsets -- even in the presence of *Id3*.

To further characterize the expression of *Id2* in γδ T cells, we utilized the Id2^{GFP} reporter mouse. The mouse was also generated with 129 ES cells (13) and subsequently backcrossed to the B6 background for more than 10 generations; however, region around the *Id2* locus still retain genetic material from the 129 background, possibly also including the putative regulatory region we identified here. In thymus, we found that *Id2* is not expressed in developing γδ T cells, but is highly expressed in their mature stage (TCRγδ$^+$CD24$^-$CD44^{high}) (20) **(Fig 4A)**. We also found that *Id2* expression is higher in the Vγ1.1$^+$Vδ6.3$^+$ γδ T cells compared to Vγ1.1$^-$Vδ6.3$^-$ γδ T cells **(Fig 4B)**. When GFP-negative γδ T cells were sorted and cultured with OP9-DL1 cells and IL-7, stimulation with anti-TCRγδ antibody further up-regulated the expression of GFP, suggesting that the expression of *Id2* may be controlled by TCR signaling **(Fig 4C)**.

We next examined the effect of *Id2* deficiency alone on the development of γδ T cells using the Id2$^{f/f}$ CD4Cre single conditional knockout model. We found that Id2$^{f/f}$ CD4Cre$^+$ mice indeed have more γδ T cells in the spleen compared to Id2$^{f/f}$ CD4Cre$^-$ mice **(Fig 4D)**. In the thymus, although the percentage and number of total γδ T cells are similar between the groups, Id2$^{f/f}$ CD4Cre$^+$ mice have more mature γδ T cell (TCRγδ$^+$CD24$^-$CD44^{high}) compared to Id2$^{f/f}$ CD4Cre$^-$ mice **(Fig 4E)**. They also have more Vγ1.1$^+$Vδ6.3$^+$ γδ T cells, although unlike in Id3$^{-/-}$ mice, these Vγ1.1$^+$Vδ6.3$^+$ γδ T cells still contribute to only a

minority of γδ T cells **(Fig 4F)**. These findings supported the hypothesis that *Id2* functions as an inhibitor of γδ T cell development, although its effect is not limited to $V\gamma1.1^+V\delta6.3^+$ γδ T cells. This inhibition effect of *Id2* is attributed to increased cell death, as mature γδ T cells from $Id2^{f/f}$ $CD4Cre^+$ mice showed decreased cell death in 7AAD/annexin V analysis but showed no difference in BrdU incorporation assays **(Fig 4G,H)**.

Id proteins control γδ T cell development through inhibition of E proteins in a developmental stage-specific manner

All evidence thus far pointed to the conclusion that *Id2* and *Id3* collaboratively act as "dual safety" in limiting the expansion of $V\delta6.3^+$ γδ T cells, and a higher, but not too high, activity of E proteins is required to permit the expansion of this population. To demonstrate that *Id2* and *Id3* indeed function in γδ T cells through inhibiting E proteins, namely E2A and HEB (21), we sought to combine different floxed alleles of E proteins and Id proteins with CD4Cre and determine whether reduction of E protein dosage can counter the effect of the loss of Id proteins **(Fig 5A)**. We found that removing any two to four of the *E2A* and *HEB* alleles can result in expansion of $V\delta6.3^+$ γδ T cells in $Id2^{f/f}$ $Id3^{f/f}$ $CD4Cre^+$ mice, although not all combinations result in the same degree of expansion. Nevertheless, this finding indicates that when both *Id2* and *Id3* are deleted in $V\delta6.3^+$ γδ T cells, it is the excessive activity of E proteins that limits the size of this population.

We next sought to investigate whether this regulation of γδ T cells by Id proteins and E proteins occurs before or after γδ lineage specification. Although $E2A^{f/f}$ $HEB^{f/f}$ $Id2^{f/f}$ $Id3^{f/f}$ $CD4Cre^+$ mice can accumulate a significant number of $V\delta6.3^+$ γδ T cells in their thymus, deletion of these four genes by LckCre, which becomes active earlier in the DN3 stage, blocked the development of $V\delta6.3^+$ γδ T cells **(Fig 5B)**. The results indicate that E protein and Id protein play different roles before and after γδ lineage specification.

Discussion

In this study, we found that the dramatic expansion of $V\gamma1.1^+V\delta6.3^+$ γδ T cells observed in $Id3^{-/-}$ mice is contingent on B6 homozygocity in a small region on chromosome 12, which possibly contains a regulatory element that leads to lower expression of the nearby *Id2* gene. Using the $Id2^f$ allele that is of 129 origin, we showed that this region alone is capable of suppressing $V\gamma1.1^+V\delta6.3^+$ γδ T cell accumulation, and this suppression is dependent on the *Id2* gene itself. However, complete loss of *Id2* and *Id3* actually reduces the $V\gamma1.1^+V\delta6.3^+$ γδ T cell population size, indicating that unrestrained E protein activity is also detrimental to these cells. We further showed that conditional knock-out of *Id2* alone was sufficient to induce a moderate expansion of γδ T cells. All of these phenomena occurred when CD4Cre was used to delete the Id and E protein genes; when the genes were deleted earlier in T cell development with LckCre, even removal of E proteins cannot restore the $V\gamma1.1^+V\delta6.3^+$ γδ T cells in *Id2* and *Id3* conditional knockout mice, emphasizing the stage-specific nature of these genetic regulations.

We propose a dual safety model to summarize the above findings **(Fig 6)**. In this model, Id3 and Id2 are differentially regulated by the TCR signals. Egr is the major transcription factor acting between the TCR signal and Id3 in T cell development (10). PLZF is a unique transcription factor involved in the development of innate-like lymphocytes such as iNKT and $V\gamma1.1^+V\delta6.3^+$ γδ T cells (22, 23). Id2 has been shown to be activated by PLZF, which is a direct target of Egr2 in iNKT cell development (24). When both Id2 and Id3 are present, they respond to the TCR signal and keep E protein activity low, and consequently prevent the expansion of $V\gamma1.1^+V\delta6.3^+$ γδ T cells. When Id3 is deleted, Id2 will assume a safety role to control E-protein activity. However, this safety role of Id2 is compromised by the hypomorphic allele of Id2 in the B6 background, allowing an increase in E protein activities

to the optimal level for driving $V\gamma1.1^+V\delta6.3^+$ γδ T cell expansion. When both Id2 and Id3 are completely deleted, E protein activity becomes too high and again limits the $V\gamma1.1^+V\delta6.3^+$ γδ T cell population.

Our study indicated that the level of E protein activity, regulated by *Id2* and *Id3* expression levels, is crucial for γδ T cell development, especially during the "maturation" stage. Both very high and very low E protein activity can limit the accumulation of γδ T cells, especially the $V\gamma1.1^+V\delta6.3^+$ γδ T cells. Since Id proteins are up-regulated by TCR signaling, a developmental restrain imposed by high Id protein level and low E activity can be interpreted as a mechanism the body uses to limit the number of γδ T cells that can recognize self antigen in the thymus, reiterating the idea that *Id2* and *Id3* are "dual safety" involved in the negative selection of γδ T cells (10). However, this "negative selection" seems to be affected by age of the animal and TCR V segment usage. The $V\gamma1.1^+V\delta6.3^+$ γδ T cells, but not other autoreactive γδ T cells, dramatically expand during the neonatal period in *Id3* deficient mice on B6 background. Why is this specific population particularly sensitive to Id protein regulation? One possibility is the presence of its cognate antigen. $V\gamma1.1^+V\delta6.3^+$ γδ T cells have been shown to recognize HSP60 of both mouse and Mycobacteria origin (25). Expression level of this antigen or other possible ligands of the $V\gamma1.1^+V\delta6.3^+$ γδ TCR may change in the thymus during development, thus making these γδ T cells specifically prone to expand during the neonatal window, unless the Id proteins prevent them from doing so. Alternatively, the expression of Id proteins in response to TCR signaling may be different between cells generated in the neonatal period versus those generated in the adult stage, and different in cells utilizing other TCR V segments; mechanisms other than Id and E proteins may be more important in restraining autoreactive γδ T cells in those conditions, so they are less affected by Id protein deletion. Nevertheless, in the *Id2* single conditional knockout mouse, γδ T cells other than those expressing Vγ1.1 and Vδ6.3 also expanded, indicating that *Id2* is broadly involved in the suppression of γδ T cell expansion.

However, our study also showed that total loss of *Id2* and *Id3* can impair γδ T cell proliferation and survival. Unrestricted E protein activity can lead to death of T cells, especially effector and memory T cells, which is well documented in the studies of peripheral CD4 and CD8 αβ T cells (26, 27). γδ T cells are considered innate-like cells, and many of them have an effector phenotype even in the thymus (1). Therefore, it is not surprising that they share the same requirement of Id protein activity with effector αβ T cells.

What is the physiological consequence of having a larger pool of autoreactive γδ T cells? The *Id3* deficient mice spontaneously develop an autoimmune disease similar to human Sjogren's syndrome (28). The large population of $V\gamma1.1^+V\delta6.3^+$ γδ T cells in these mice is potentially involved in the pathogenesis. However, previous report also showed that these cells can play a role in suppressing tissue inflammation (3). More tests are required to further clarify the impact of the expanded γδ T cell population in mice with Id protein deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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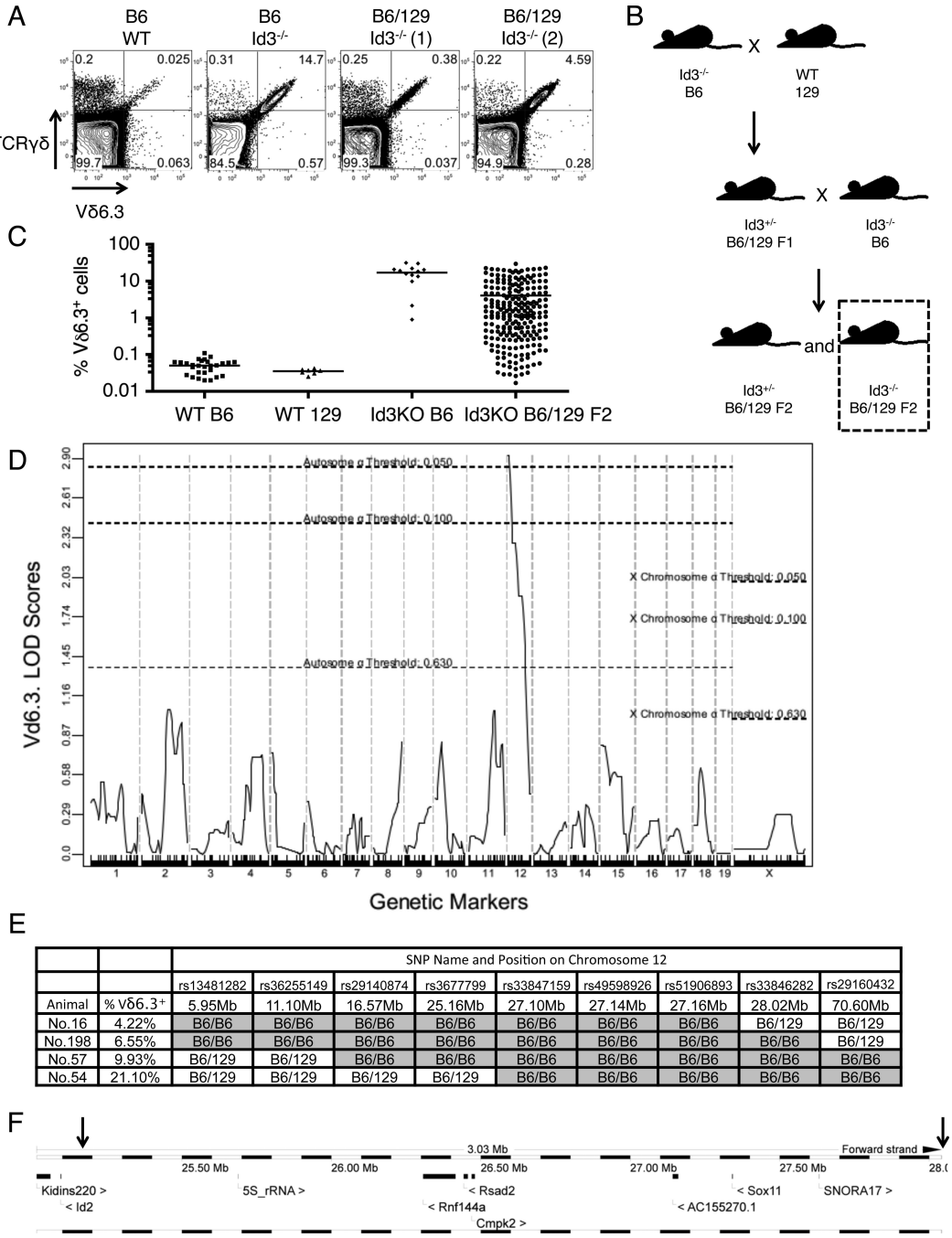


		rs13481282	rs36255149	rs29140874	rs3677799	rs33847159	rs49598926	rs51906893	rs33846282	rs29160432
Animal	% Vδ6.3+	5.95Mb	11.10Mb	16.57Mb	25.16Mb	27.10Mb	27.14Mb	27.16Mb	28.02Mb	70.60Mb
No.16	4.22%	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/129	B6/129
No.198	6.55%	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/129
No.57	9.93%	B6/129	B6/129	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6
No.54	21.10%	B6/129	B6/129	B6/129	B6/129	B6/B6	B6/B6	B6/B6	B6/B6	B6/B6

Figure 1.

A single locus on mouse chromosome 12 strongly influences the development of Vδ6.3$^+$ γδ T cells in *Id3*-deficient mice. **(A)** Deficiency of *Id3* induces the accumulation of a large population of Vδ6.3$^+$ γδ T cells in the thymus of mice with pure B6 background. However, this phenomenon persists in some but is absent in other mice with a B6/129 mix background, as two representative mice are showing here. **(B)** A breeding scheme was established to dissect potential modifying gene(s) in the B6 and 129 genetic backgrounds. Only F2 B6/129 hybrid Id3$^{-/-}$ mice were used in the linkage analysis. **(C)** Percentages of Vδ6.3$^+$ γδ T cells among total thymocytes were scored for individual mice of indicated genotype group. Each dot represents one mouse. Horizontal line indicates mean of the

genotype group. **(D)** SNP analysis of the B6/129 hybrid Id3$^{-/-}$ mice showed that one location on chromosome 12 is strongly correlated with the presence of more than 1% of Vδ6.3^{+} γδ T cells in the thymus. **(E)** Detailed SNP analysis of four mice with more than 1% of Vδ6.3^{+} γδ T cells in the thymus showed a linkage to B6 homozygocity within a 3 Mb region on chromosome 12. **(F)** A map of known features around the critical region on chromosome 12; arrows indicate the border of the region (25.16Mb-28.02Mb) as determined in **E**. Note that *Id2* and *Kidins220* are immediately outside of the border of this region.

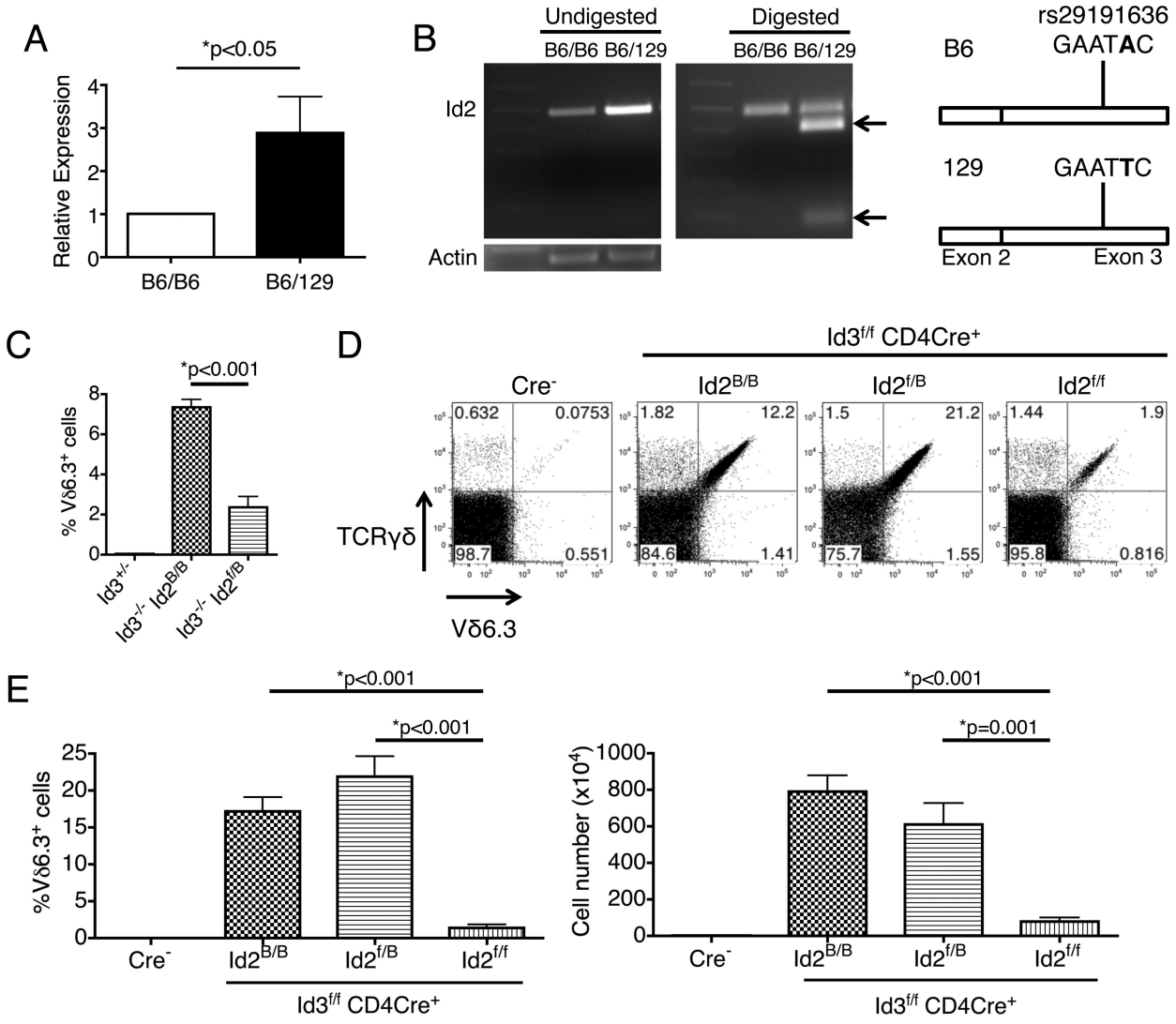


Figure 2.

Id2 is a major modifier of Vδ6.3$^+$ γδ T cell development in *Id3*-deficient mice. **(A)** QPCR analysis showed that *Id2* mRNA expression in Vδ6.3$^+$ γδ T cells from Id3$^{-/-}$ mice with B6 background on the chromosome 12 region encompassing *Id2* is lower than those with B6/129 mix background. n=3 for independent sorting of each genotype group. **(B)** Restriction enzyme analysis of the SNP marker rs29191636 within the exon 3 of the *Id2* gene. EcoRI digestion of *Id2* cDNA made from Vδ6.3$^+$ γδ T cells with B6/129 mix background generated abundant product specific to the 129 allele (marked by two arrows), indicating that the higher *Id2* expression in these cells came from that allele. Data representative of 3 experiments. **(C)** Replacement of one copy of the *Id2* B6 allele with the Id2^f allele of 129 origin is sufficient to reduce the population size of Vδ6.3$^+$ γδ T cells. Id2^B indicates the wild type *Id2* allele in B6 background. n≥3 for each group. **(D)** Analysis of Vδ6.3$^+$ γδ T cells with various combinations of Id2^B and Id2^f alleles on Id3$^{f/f}$ CD4Cre$^+$ background. Data representative of 3 mice in each group. **(E)** Percentage and number of Vδ6.3$^+$ γδ T cell in the thymus of genotype each group shown in D. N≥4 in each group. All error bars indicate SD.

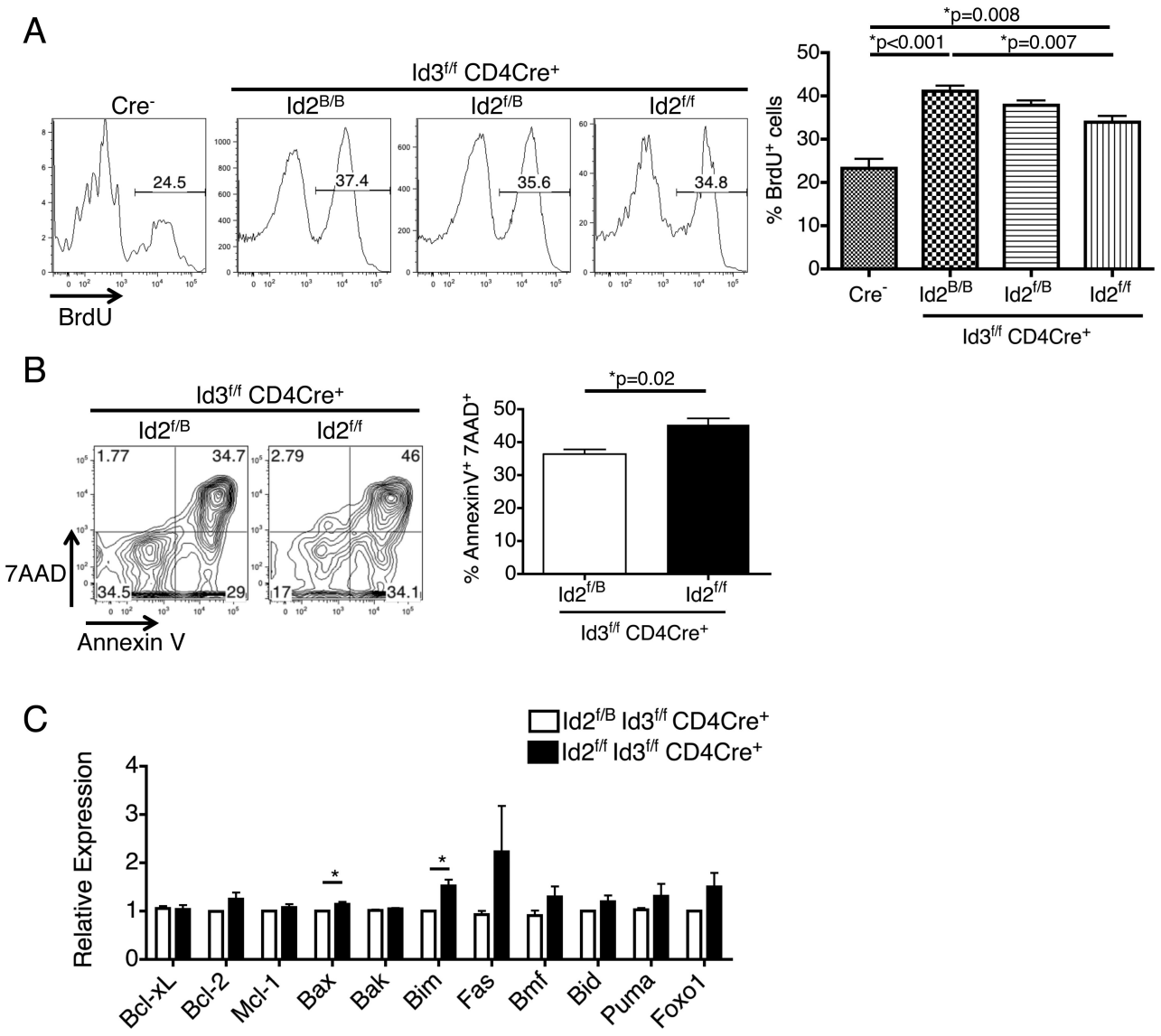


Figure 3.

Conditional knockout of both *Id2* and *Id3* impairs the proliferation and survival of Vδ6.3$^+$ γδ T cells. **(A)** In the neonatal thymus, Vδ6.3$^+$ γδ T cells from Id3$^{f/f}$ CD4Cre$^+$ mice are more highly proliferative than the Cre$^-$ controls as shown by BrdU incorporation assay, regardless of their *Id2* genotype. However, cells from the Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ mice show a small but significant decrease in BrdU$^+$ cell percentage compared to those from Id2$^{B/B}$Id3$^{f/f}$CD4Cre$^+$ mice. n=3 for each group. **(B)** Vδ6.3$^+$ γδ T cells were sorted from the thymus of neonatal mice and cultured for 24 hours. Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ cells showed increased cell death by 7AAD and Annexin V staining compared to Id2$^{f/B}$ Id3$^{f/f}$ CD4Cre$^+$ cells. n=3 in each group. **(C)** QPCR analysis of a panel of cell death-related genes showed that Id2$^{f/f}$ Id3$^{f/f}$ CD4Cre$^+$ Vδ6.3$^+$ γδ T cells express more mRNA of pro-apoptotic genes *Bim* and *Bax*. n=3 in each group. *p<0.05. All error bars indicate SD.

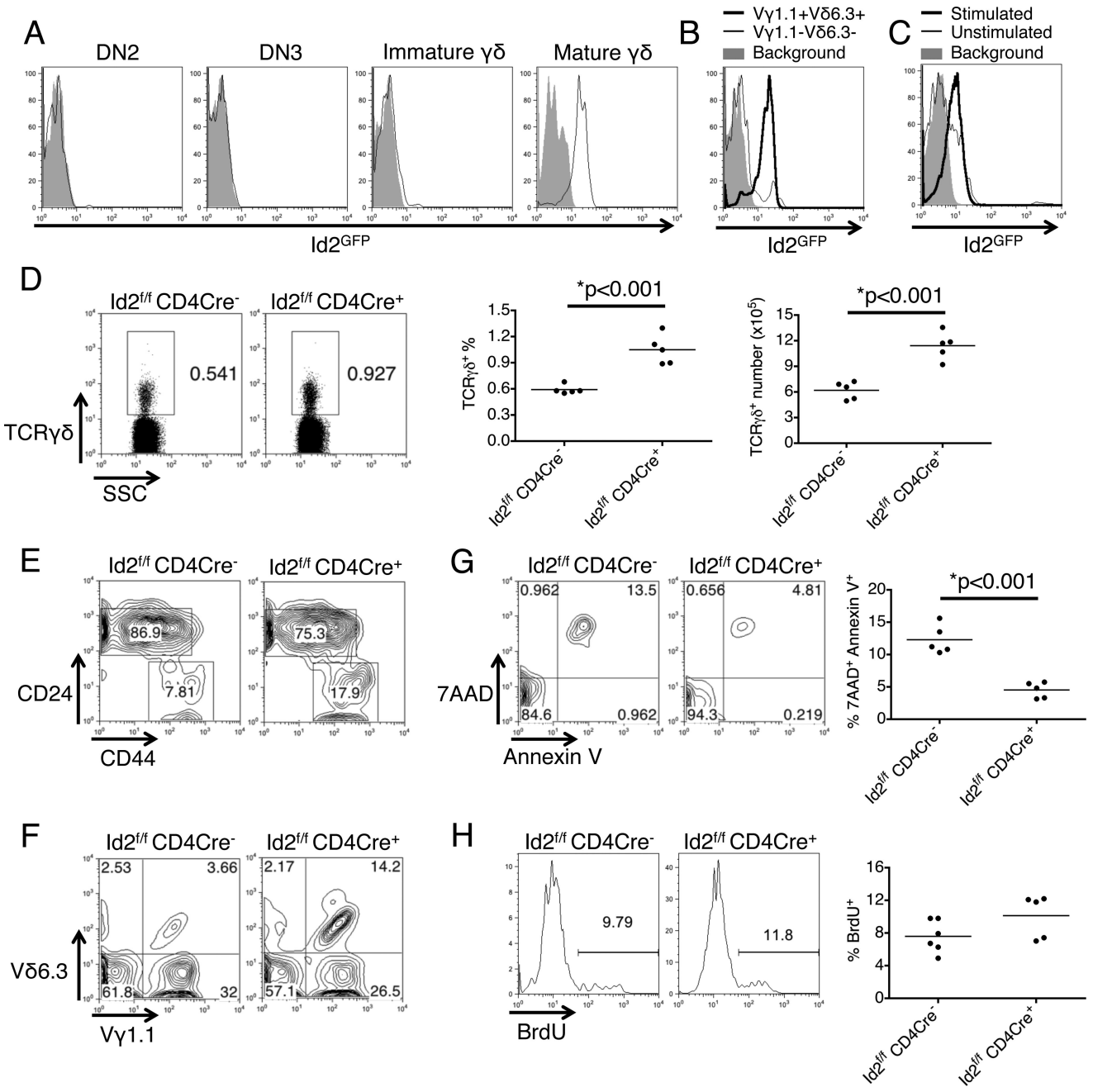


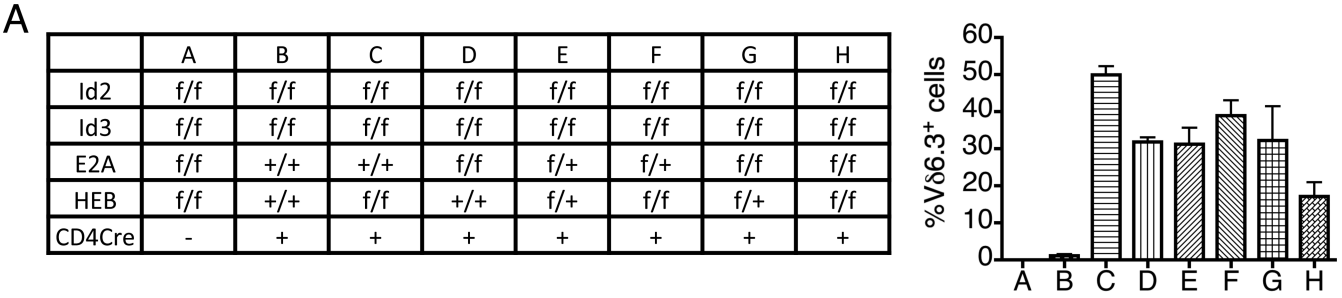
Figure 4.

Conditional knockout of *Id2* alone results in expansion of γδ T cells. **(A)** Examination of *Id2* expression in developing γδ T cells in the thymus with an $Id2^{GFP}$ reporter showed that *Id2* is up-regulated at the mature stage. DN2: $Lin^{-}CD25^{+}CD44^{+}$. DN3: $Lin^{-}CD25^{+}CD44^{-}$. Immature: $TCR\gamma\delta^{+}CD24^{+}CD44^{low}$. Mature: $TCR\gamma\delta^{+}CD24^{-}CD44^{high}$. **(B)** *Id2* expression is higher in the $V\gamma1.1^{+}V\delta6.3^{+}$ cells than in other γδ T cells. **(C)** *In vitro* culturing of sorted $Id2^{GFP}$ negative γδ T cells from the thymus for 5 days with IL-7 and anti-TCR γδ stimulation resulted in more significant up-regulation of *Id2* compared to culturing with IL-7 alone. For A-C, data representative of 3 mice in each group. **(D)** $Id2^{f/f}$ $CD4Cre^{+}$ mice have more γδ T cells in the spleen compared to $Id2^{f/f}$ $CD4Cre^{-}$ mice. Bar graphs show the percentage and number of γδ T cells in the spleen of mice in each group. Each dot represents

one mouse. **(E)** $Id2^{f/f}$ $CD4Cre^{+}$ mice have a higher percentage of $CD24^{-}CD44^{high}$ mature γδ T cells in the thymus. Pre-gated on $TCR\gamma\delta^{+}$ cells. **(F)** $Id2^{f/f}$ $CD4Cre^{+}$ mice have a higher percentage of $V\gamma1.1^{+}V\delta6.3^{+}$ γδ T cells in the thymus. Pre-gated on $TCR\gamma\delta^{+}$ cells. **(G)** The mature thymic γδ T cells from $Id2^{f/f}$ $CD4Cre^{+}$ mice show decreased cell death by 7AAD and Annexin V staining. **(H)** The mature thymic γδ T cells from $Id2^{f/f}$ $CD4Cre^{+}$ mice show similar proliferation rate to $Id2^{f/f}$ $CD4Cre^{-}$ mice in BrdU incorporation assay. Data representative of 3 mice in each group in E and F.

A

	A	B	C	D	E	F	G	H
Id2	f/f	f/f	f/f	f/f	f/f	f/f	f/f	f/f
Id3	f/f	f/f	f/f	f/f	f/f	f/f	f/f	f/f
E2A	f/f	+/+	+/+	f/f	f/+	f/+	f/f	f/f
HEB	f/f	+/+	f/f	+/+	f/+	f/f	f/+	f/f
CD4Cre	-	+	+	+	+	+	+	+



B

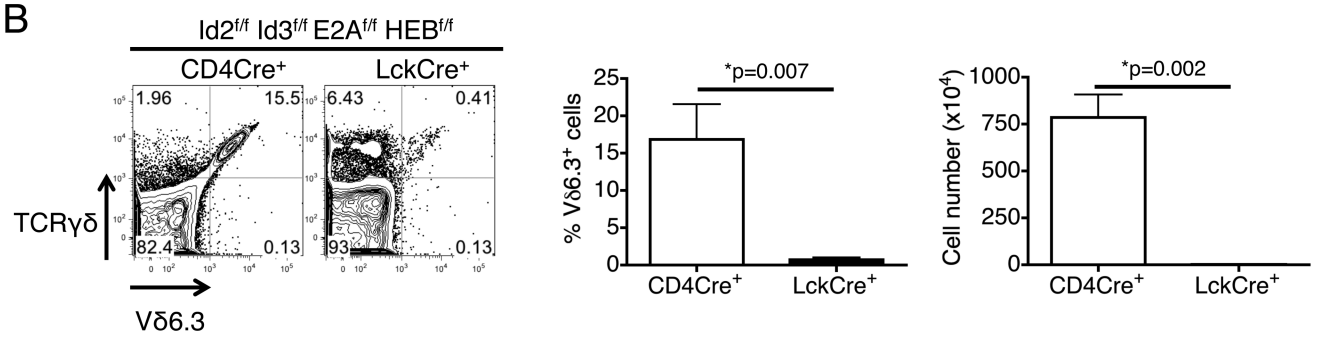


Figure 5.

The effect of Id proteins on Vδ6.3$^+$ γδ T cells is mediated by E proteins and is developmental stage-specific. **(A)** Although conditional knockout of both *Id2* and *Id3* limits the accumulation of Vδ6.3$^+$ γδ T cells in the thymus, further deletion of *HEB* and/or *E2A* can restore the accumulation of those cells. N≥3 for each group. **(B)** Deletion of all alleles of *HEB*, *E2A*, *Id2* and *Id3* by CD4Cre can induce accumulation of Vδ6.3$^+$ γδ T cells, but deletion by LckCre fails to induce a similar phenotype. N≥3 for each group. All error bars indicate SD.

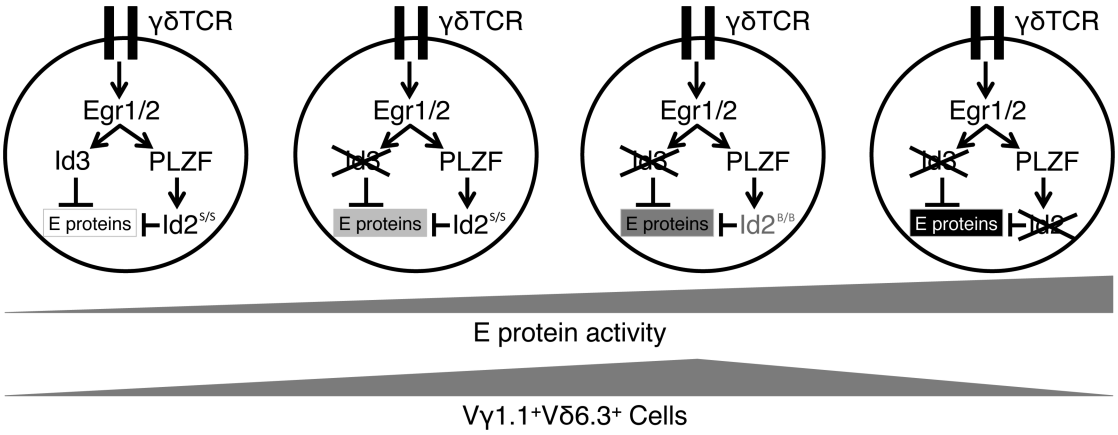


Figure 6.

A schematic diagram of Vγ1.1^{+}Vδ6.3^{+} γδ T cell developmental control by Id2 and Id3. In the developing thymus, γδ T cells that express the Vγ1.1 and Vδ6.3 TCR segments receive strong TCR signaling, up-regulating Id2 and Id3 through Egr1/2 and PLZF. The Id proteins inhibit activity of E proteins, affecting the survival and proliferation of Vγ1.1^{+}Vδ6.3^{+} γδ T cells. When Id3 is present, and Id2 is expressed from a more active allele, such as the one from the 129 genetic background (Id2^{s}, “strong”), E protein activity is very low and Vγ1.1^{+}Vδ6.3^{+} γδ T cell population size is small. If Id3 is absent, and Id2 is expressed from a less active allele, such as the one from the B6 background (Id2^{B}, “B6”), E protein activity becomes higher and the Vγ1.1^{+}Vδ6.3^{+} γδ T cells expand dramatically. However, if both Id2 and Id3 are completely absent, E protein activity becomes too high and again impairs the survival and proliferation of Vγ1.1^{+}Vδ6.3^{+} γδ T cells, limiting its population size.