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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*J Immunol* 2014; 192:1055-1063; Prepublished online 30 December 2013; doi: 10.4049/jimmunol.1302694
http://www.jimmunol.org/content/192/3/1055
Id3 and Id2 Act as a Dual Safety Mechanism in Regulating the Development and Population Size of Innate-like γδ T Cells

Baojun Zhang,1 Yen-Yu Lin,1 Meifang Dai, and Yuan Zhuang

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 Vγ1.1⁺Vδ6.3⁺ 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 Vγ1.1⁺Vδ6.3⁺ 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 homolog of Id3, to be the major modifier of Id3 in limiting Vγ1.1⁺Vδ6.3⁺ T cell expansion. The Vγ1.1⁺Vδ6.3⁺ 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 Vγ1.1⁺Vδ6.3⁺ T cells because of 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 Vγ1.1⁺Vδ6.3⁺ T cells are mediated by titration of E protein activity, because removing one or more copies of E protein genes can restore Vγ1.1⁺Vδ6.3⁺ 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. The Journal of Immunology, 2014, 192: 1055–1063.

A subset of T lymphocytes generated in the thymus, γδ T cells function between the innate and adaptive immune system. They have features of the adaptive immune system, such as the expression of variable rearranged γδ TCRs, 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 internal organs, mucosal surfaces, 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 TCR belong to a unique subset. In mice, these T cells are produced in large numbers in the neonatal thymus (5) and are capable of rapidly producing multiple cytokines, including IFN-γ 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 TCR 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 because of their unique ability among γδ T cells to produce Th2-like cytokines (as reviewed by Carding and Egan [3]). The population size of Vγ1.1⁺Vδ6.3⁺ 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 Vγ1.1⁺Vδ6.3⁺ 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 TCR genes, the surface expression of γδ TCR can send a strong signal into the cell and upregulate Id3, promoting the cell to adopt a γδ T cell fate (10). However, Id3 also plays a distinct inhibitory role controlling the development of Vγ1.1⁺Vδ6.3⁺ γδ T cells because this population is dramatically expanded in Id3-deficient mice. More interesting, 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 are also critical in the development of Vγ1.1⁺Vδ6.3⁺ γδ T cells in the absence of Id3.
Although 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 Vγ1.1V66.3+ 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 Vγ1.1V66.3+ 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 Vγ1.1V66.3+ 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 Vγ1.1V66.3+ subset. Paradoxically, if both Id2 and Id3 are completely deleted, the Vγ1.1 V66.3+ γδ T cells actually fail to accumulate, possibly because of 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(+/– (13), Id2+/+ (14), Id3+/+ (15), E2A(+/– (16), HEB(+/– (17), and LckCre transgenic (18) mice have been described previously and all backcrossed to C57BL/6J for more than 10 generations. 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 specific pathogen-free 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 Abs 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 TCRα/β (2.11), anti-mouse CD24 (M1/69), and anti-mouse TCRβ (H57-597) purchased from Biolegend. PE anti-mouse V6 6.3/2 (8F4H7B7) Ab, Annexin V, and APC BrdU Flow Kit were purchased from BD Biosciences. 7-Aminoactinomycin D (7AAD) 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 containing 5% bovine calf serum). A total of 1 × 10^6 cells were stained with Abs 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 JQTL mapping program (version 1.3; http://churchill.jax.org/software/jqtl.shtml), and genomic regions with significant linkage to the expansion of Vγ1.1/V66.3+ 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+ V6 6.3+ cells were sorted from mouse thymus, and total RNA was extracted with RNAqueous micro kit (Life Technologies). Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (Life Technologies). SYBR-based real-time PCR was performed to quantitatively compare gene expression, normalized to β-actin. Quantitative PCR (QPCR) primer sequences are available upon request.

RFLP analysis

A 481-bp fragment was PCR amplified from Id2 cDNA made from thymic TCRγδ+ V6 6.3+ cells. The PCR product was digested with EcoRI. The 481-bp 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 393-bp fragment and one 88-bp fragment.

In vivo BrdU incorporation assays

One milligram of BrdU was i.p. injected into each mouse 15 (for Id3+/– CD4Cre+ versus Id2+/+ CD4Cre+ mice experiments) or 4 h (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’s protocol.

Cell death analysis

For Id3+/– CD4Cre+ versus Id2+/+ CD4Cre+ mice experiments, thymocytes were harvested and directly analyzed with 7AAD/Annexin V for cell death according to manufacturer’s protocol (BD Biosciences). For Id2+/+ Id3+/– CD4Cre+ versus Id2+/+ Id3+/+ CD4Cre+ mice experiments, TCRγδ+ V6 6.3+ cells were first sorted from mouse thymus and cultured in RPMI 1640 medium supplemented with 10% FBS and 55 μM 2-ME for 24 h before 7AAD/Annexin V analysis.

Statistical analysis

Sample data were compared using Student t test, and a p value < 0.05 was considered significant.

Results

Backcross mapping identifies a single locus that modulates numbers of V66.3+ cells in Id3 knockout mice

We analyzed the thymus of Id3-deficient mice on B6 or B6/129 mixed background and showed that the population of Vγ1.1 V66.3+ γδ T cells is consistently large in mice with B6 background but variable in mice with B6/129 mix background (Fig. 1A, Supplemental Fig. 1A). Because V66.3 usage is uniquely associated with the Vγ1.1/V66.3+ population found in Id3-deficient mice (11), V66.3 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 V66.3+ γδ T cell percentage in the thymus. Among all the 226 Id3+/– F2 mice analyzed, we found a wide range of distribution in V66.3+ γδ T cell percentage (Fig. 1C). As a comparison, most Id3+/– mice on B6 background have >1% V66.3+ γδ T cells among thymocytes (Fig. 1C). This result suggests the existence of one or more possible Id3 modifier genes, with the 129 allele being dominant (inhibit V66.3+ γδ T cell expansion) and the B6 allele being recessive (permit V66.3+ γδ T cell expansion). 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 V66.3⁺ γδ T cells (logarithm of odds score > 2; Fig. 1D). Focusing on one SNP on chromosome 12 location 30 Mb, we analyzed additional 138 $Id^3^{-/-}$ F2 mice and showed that B6/B6 homozygosity of this location was significantly correlated with the accumulation of V66.3⁺ γδ T cells (Supplemental Fig. 1B). We further analyzed these 138 $Id^3^{-/-}$ F2 mice, determining their genotype of two SNP markers on chromosome 12 (location around 3 Mb and 30 Mb) flanking the
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 nonsynonymous changes or splice-site SNPs for Sox11, Cmpk2, Rsad2, and Id2 genes. Several nonsynonymous 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 (Supplemental Fig. 2A, 2B).

We considered the alternative possibility that variation in noncoding 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 25.16–28.02 Mb. 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, Supplemental Fig. 2C). To verify that the increased expression comes from the 129 Id2 allele, we performed RFLP analysis of the Id2 cDNA made from the γδ T cells. The 129 Id2 allele contains an EcoRI restriction site in the 3' untranslated region that 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 25.16 and 28.02 Mb, acts in cis to control Id2 expression. The 129 version of the element may induce a higher

![Image](http://www.jimmunol.org/Downloaded_from)
level of Id2 expression than the B6 version, and this higher Id2 level may inhibit the expansion of V66.3+ γδ T cells in a dominant manner.

To further examine this hypothesis, we took advantage of the Id2f allele (as compared with Id2h, 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 >10 generations, the region on chromosome 12 around Id2, including location 25.16–28.02 Mb, 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 Id2f/+ mouse on B6 background, we can specifically investigate the role of this region on the development of V66.3+ γδ T cells. We found that introducing one copy of the Id2f allele was sufficient to significantly suppress the accumulation of V66.3+ γδ T cells (Fig. 2C). To determine whether it is Id2 itself that is limiting the development of V66.3+ γδ T cells, we knocked out Id2 and Id3 in T cells specifically with CD4Cre. The Id2f/Bh Id3f/+ CD4Cre+ mice behaved similarly to Id2f/+ mice, showing significant accumulation of V66.3+ γδ T cells (Fig. 2D). However, unlike the Id2f/Bh Id3f/+ mice, the 129 genetic material in the Id2f/Bh Id3f/+ CD4Cre+ mice could not suppress the accumulation of V66.3+ 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 located 25.16–28.02 Mb from the 129 background may influence the expression of Id2, thus inhibiting the accumulation of V66.3+ γδ T cells together with Id3.

We hypothesized that double deletion of Id2 and Id3 with CD4Cre might lead to further increase of the V66.3+ γδ T cells. To our surprise, Id2f/Bh Id3f/+ CD4Cre+ mice actually have very few of these cells (Fig. 2D, 2E). Because the V66.3+ γδ T cells proliferate vigorously during the neonatal stage, we examined thymocytes from 7-d-old mice and found that, regardless of the Id2 genotype, V66.3+ γδ T cells from Id3f/+ CD4Cre+ mice were more highly proliferative than those from the Cre− controls, but the proliferation is slightly attenuated in Id2f/Bh Id3f/+ CD4Cre+ mice (Fig. 3A). When we cultured the cells in vitro for 24 h, we found that cells from Id2f/Bh Id3f/+ CD4Cre+ mice showed more rapid cell death than those from Id2f/Bh Id3f/+ 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 upregulated in Id2f/+ Id3f/+ CD4Cre+ cells (Fig. 3C). These results indicate that complete removal of both Id2 and Id3 proteins can actually be inhibitory for V66.3+ γδ T cell survival and proliferation, implying that the maximal output of V66.3+ γδ T cells requires an optimal level of Id proteins.

Id2 functions as an inhibitor of γδ T cell development

Because complete knockout of both Id2 and Id3 is detrimental to the accumulation of V66.3+ γδ T cells, one possible explanation for the expansion of these cells in Id2f/Bh Id3f−/+ 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 V66.3+ 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 used the Id2Fyp reporter mouse. The mouse was also generated with 129 embryonic stem cells (13) and subsequently backcrossed to the B6 background for >10 generations; however, regions around the id2 locus still retain genetic material from the 129 background, possibly also including the putative regulatory region we identified in this study. In thymus, we found that Id2 is not expressed in developing γδ T cells but is highly expressed in their mature stage (TCRγδCD4+ CD44high) (20) (Fig. 4A). We also found that Id2 expression is higher in the Vγ1.1+V66.3+ γδ T cells compared with Vγ1.1−V66.3− γδ T cells (Fig. 4B). When

**FIGURE 3.** Conditional knockout of both Id2 and Id3 impairs the proliferation and survival of V66.3+ γδ T cells. (A) In the neonatal thymus, V66.3+ γδ T cells from Id3f/+ 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 Id2f/+ Id3f/+ CD4Cre+ mice show a small but significant decrease in BrdU+ cell percentage compared with those from Id2f/+ Id3f/+ CD4Cre+ mice. n=3 for each group. (B) V66.3+ γδ T cells were sorted from the thymus of neonatal mice and cultured for 24 h. Id2f/+ Id3f/+ CD4Cre+ cells showed increased cell death by 7AAD and Annexin V staining compared with Id2f/+ Id3f/+ CD4Cre+ cells. n=3 in each group. (C) QPCR analysis of a panel of cell death–related genes showed that Id2f/+ Id3f/+ CD4Cre+ V66.3+ γδ T cells express more mRNA of proapoptotic genes Bim and Bax. n=3 in each group. *p<0.05. All error bars indicate SD.
GFP+ γδ T cells were sorted and cultured with OP9-DL1 cells and IL-7, stimulation with anti-TCRγδ Ab further upregulated 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 Id2f/f CD4Cre single conditional knockout model. We found that Id2f/f CD4Cre+ mice indeed have more γδ T cells in the spleen compared with Id2f/f CD4Cre− mice (Fig. 4D). In the thymus, although the percentage and number of total γδ T cells are similar between the groups, Id2f/f CD4Cre+ mice have more mature γδ T cell (TCRγδ+CD24−CD44hi) compared with Id2f/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 Vγ1.1′ Vδ6.3′ γδ T cells. This inhibition effect of Id2 is attributed to increased cell death, as mature γδ T cells from Id2f/f CD4Cre+ mice showed decreased cell death in 7AAD/Annexin V analysis but showed no difference in BrdU incorporation assays (Fig. 4G, 4H).

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
V66.3⁺ 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 HEV (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 HEV alleles can result in expansion of V66.3⁺ γδ T cells in Id3⁻ Id2⁺ 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 V66.3⁺ 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⁻ Id2⁺ CD4Cre⁺ mice can accumulate a significant number of V66.3⁺ 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 V66.3⁺ γδ T cells (Fig. 5B). The results indicate that E protein and Id protein play different roles before and after γδ lineage specification. Collectively, these data support the idea that Id3 and Id2 function downstream of the TCR signaling to regulate E protein activity, and the Vγ1.1⁺V66.3⁺ cell development is driven by an optimal level of E protein activity (Fig. 6).

Discussion

In this study, we found that the dramatic expansion of Vγ1.1⁺V66.3⁺ γδ T cells observed in Id3⁻/⁻ mice is contingent on B6 homozygosity 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⁺ allele that is of 129 origin, we showed that this region alone is capable of suppressing Vγ1.1⁺ V66.3⁺ γδ T cell accumulation, and this suppression is dependent on the Id2 gene itself. However, complete loss of Id2 and Id3 actually reduces the Vγ1.1⁺V66.3⁺ γδ T cell population size, indicating that unrestrained E protein activity is also detrimental to these cells. We further showed that conditional knockout 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 could not restore the Vγ1.1⁺V66.3⁺ 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 role of Id3 and Id2 in the control of Vγ1.1⁺V66.3⁺ cell development (Fig. 6).
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 Vγ1.1Vδ6.3+ 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 Vγ1.1+ Vδ6.3+ 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 Vγ1.1Vδ6.3+ γδ T cell expansion. When both Id2 and Id3 are completely deleted, E protein activity becomes too high and again limits the Vγ1.1Vδ6.3+ 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 Vγ1.1Vδ6.3+ γδ T cells. Because Id proteins are upregulated by TCR signaling, a developmental restraint 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-Ag 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 Vγ1.1Vδ6.3+ 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 Ag. Vγ1.1Vδ6.3+ γδ T cells have been shown to recognize HSP60 of both mouse and Mycobacteria origin (25). Expression level of this Ag or other possible ligands of the Vγ1.1Vδ6.3+ 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 using 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 Sjögren’s syndrome (28). The large population of Vγ1.1Vδ6.3+ γδ T cells in these mice is potentially involved in the pathogenesis. However, a 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.

Disclosures

The authors have no financial conflicts of interest.

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