Inhibitor of DNA Binding 3 Limits Development of Murine Slam-Associated Adaptor Protein-Dependent “Innate” γδ T cells

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Abstract

Background: Id3 is a dominant antagonist of E protein transcription factor activity that is induced by signals emanating from the αβ and γδ T cell receptor (TCR). Mice lacking Id3 were previously shown to have subtle defects in positive and negative selection of TCRαβ+ T lymphocytes. More recently, Id3−/− mice on a C57BL/6 background were shown to have a dramatic expansion of γδ T cells.

Methodology/Principal Findings: Here we report that mice lacking Id3 have reduced thymocyte numbers but increased production of γδ T cells that express a Vγ1.1 Vδ6.3+ receptor with restricted junctional diversity. These Vγ1.1 Vδ6.3+ T cells have multiple characteristics associated with “innate” lymphocytes such as natural killer T (NKT) cells including an activated phenotype, expression of the transcription factor PLZF, and rapid production of IFNg and interleukin-4. Moreover, like other “innate” lymphocyte populations, development of Id3−/− Vγ1.1 Vδ6.3+ T cells requires the signaling adapter protein SAP.

Conclusions: Our data provide novel insight into the requirements for development of Vγ1.1 Vδ6.3+ T cells and indicate a role for Id3 in repressing the response of “innate” γδ T cells to SAP-mediated expansion or survival.

Introduction

γδ T lymphocytes bearing αβ or γδ T cell receptors (TCR) develop in the thymus from a common progenitor cell pool. Most cells in the adult thymus express the co-receptor molecules CD4 and CD8 and represent an intermediate stage in αβ T cell development that has undergone productive TCRαβ rearrangement and is in the process of TCRαβ rearrangement. Alter expression of a functional TCRα CD4+CD8+ (double positive, DP) cells undergo negative or positive selection and become single positive (SP) cells [1]. In contrast, the earliest T cell progenitors, and γδ T cells, are CD4−CD8− (double negative, DN) and can be divided into four stages based on expression of CD117 (c-kit) and CD25; DN1, (c-kit+)CD25−, DN2 (c-kit−CD25+), DN3 (c-kit−CD25−) and DN4 (c-kit−CD25−) [2]. DN1 and DN2 cells are the most immature T cell progenitors and are not yet fully committed to T cell differentiation [3,4]. Rearrangement of TCR loci initiates at the DN2 stage but is most prevalent in DN3 cells [5,6]. DN3 cells that rearrange and express TCRβ undergo β-chain selection and progress to the DN4 stage before becoming DP [7]. In contrast, cells that rearrange functional Tcra and Tcrg genes diverge from the αβ pathway and become DN γδ T cells [2]. The stage at which the αβ and γδ T cell lineages diverge remains controversial [8,9,10,11,12].

During ontogeny, the variable gene segments of the Tcra and Tcrg genes are rearranged in ordered waves. The first wave occurs around embryonic day 13 and includes rearrangement of Vγ3 and Vδ1 and is followed by rearrangement of Vγ4 [13,14] (Nomenclature according to [15]). These receptors contain limited diversity at the junction of the V, diversity (D), and joining (J) segments [16,17] in part because terminal deoxyxyunucleotidyl transferase (TdT), a polymerase that adds non-templated nucleotides, is absent from embryonic cells [18,19]. Consequently, the first γδ T cells express invariant Vγ3/Vδ1 or Vγ4/Vδ1 TCRs and home specifically to the epidermis or the epithelium of the reproductive tract and the tongue, respectively [20]. In contrast to the embryo, the adult thymus rearranges Vγ1.1, Vγ2 and Vγδ and generates receptors with extensive junctional diversity, creating a highly diverse γδ TCR repertoire [21]. Interestingly, a subset of γδ T cells with an invariant Vγ1.1 Vδ6.3+ TCR has been described that resides in the adult thymus, spleen, and liver [22]. These γδ T cells develop from

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late embryonic precursors and expand during neonatal life [23]. V_{y1}1.1 V_{66.3}^{+} T cells share multiple characteristics with natural killer (NK) T cells including expression of the activation markers CD44, and NK1.1, and low expression of the immature T lymphocyte marker CD24. Moreover, both NK1.1 and V_{y1}1.1 V_{66.3}^{+} T cells secrete IFNγ and IL4 rapidly after stimulation in vitro [24]. These findings led to the hypothesis that NK1.1 and V_{y1}1.1 V_{66.3}^{+} T cells represent innate branches of the Tβ and γδ T cell lineages, respectively [25]. The presence of an invariant receptor on these two T cell subsets is consistent with the hypothesis that the functional characteristics of these “innate-like” cells are determined in part via selection by endogenous ligands.

T cell development is intimately linked to activity of the E protein transcription factors E2A and HEB [26,27,28]. E proteins proper two T cell subsets is consistent with the hypothesis that the functional activity of CD3e (a component of the TCR signalling complex) on DN3 thymocytes induces Id3 through a MAP kinase-dependent pathway [32]. Mice lacking Id3 show mild defects in positive selection similar to those observed in mice lacking the TEC kinase Itk, which activates the MAP kinases Erk1 and Erk2 [33,34]. MAP kinase signalling is also important for proper γδ T cell development and Id3 is highly expressed in γδ T cells, although published data suggest that Id3 is not essential for γδ T cell development in mice expressing the KN6 [V_{γ7}^{a} V_{δ5}^{a}] transgene [10]. Surprisingly however, it was reported that Id3−/− mice have an increased number of γδ T cells and it was hypothesized that Id3 functions in DN3 cells to prevent Treg or Tcrg rearrangements in cells expressing a functional TCRβ [35].

Here we report that the elevated number of γδ T cells in Id3−/− mice is a consequence of an expanded population of V_{y1}1.1 V_{66.3}^{+} T cells. Id3−/− V_{y1}1.1 V_{66.3}^{+} T cells, like their wild-type (WT) counterparts, primarily develop from late embryonic or neonatal progenitors rather than adult DN3 cells. These γδ T cells have many of the characteristics of NK T cells previously noted, and we confirm that Id3−/− V_{y1}1.1 V_{66.3}^{+} T cells express the transcription factor promyelocytic leukemia zinc finger (PLZF) protein [36], a molecular determinant of the NK1.1 cell fate [37,38] and their development required the Signaling lymphocyte adaptor molecule (Slam)-associated Adaptor Protein (SAP) [39]. Importantly, deletion of SAP overcomes all apparent thymic alterations in Id3−/− mice including the increased number of γδ T cells and the reduced thymic cellularity, whereas deletion of Tcrg had no effect on thymic cellularity. These observations indicate that Id3 plays a role in preventing expansion or survival of this SAP-dependent lymphocyte. Taken together, our data demonstrate that Id3 functions to limit the development of SAP-dependent “innate-like” γδ T cells.

**Results**

**Development of CD4− and CD8− γδ T Cells in Id3−/− Mice**

While investigating the thymic phenotype of Id3−/− mice we discovered that the number of γδ T cells is increased by approximately 8-fold (range 3- to 15-fold) compared to Id3+/+ mice (Fig. 1A and B). In contrast, the frequency of TCRβ+ cells was similar among Id3+/+ and Id3−/− thymocytes, although the number of TCRβ+ cells is decreased in the absence of Id3 since Id3−/− mice have a 3-fold decrease in thymocyte numbers (Fig. 1A and Fig. S1). Further analysis revealed that a large portion of Id3−/− TCRγ+ cells express CD4 or CD8 (Fig. 1C). Compared to Id3+/+ mice, Id3−/− mice have an increased number of CD4 (80-fold) and CD8 (70-fold) TCRγ+ cells as well as DN (5.5-fold) and DP (5-fold) TCRγ+ cells (Fig. 1D). Id3−/− TCRγ+ cells express CD8 as a CD8αα homodimer as opposed to the CD8αβ heterodimer expressed by TCRβ/CD8α cells (Fig. 1E). Importantly, Id3−/− TCRγ+ cells expressed significantly more mRNA for the transcription factor Sox13 than DP thymocytes indicating that these are bona fide γδ T cells [40] (Fig. 1F). In the spleen there is also a large population of TCRγ+ cells expressing CD4 or CD8αβ that is markedly elevated compared to Id3+/+ mice (Fig. S2). Taken together these data indicate that Id3 limits development of γδ T cells, in particular, γδ T cells expressing CD4 or CD8.

**Id3−/− γδ T Cells Have an Activated or “Innate-Like” Phenotype**

In light of our observations that Id3−/− γδ T cells expressed CD4 and CD8, we characterized these cells for expression of multiple cell surface proteins. In the thymus, the majority of Id3−/− DN TCRγ+ cells had high expression of CD122, NK1.1 and CD44 and low expression of CD24 compared with Id3+/+ TCRγ+ cells (Fig. 2A and B). This phenotype is associated with activation of γδ and γδ T cells [41,42,43]. Notably, a majority of the Id3−/− NK1.1 TCRγ+ cells expressed TCRγ at low levels (Fig. 2B). A subset of γδ T cells expressing NK1.1 with low expression of TCRγ6 is present in the spleen of WT mice and presumably represent activated γδ T cells [42]. Similar to Id3−/− DN TCRγβ+ cells, a portion of Id3−/− CD4 and CD8 γδ T cells had these activation markers, although the CD4 cells had lower levels of CD122 and only a small portion expressed NK1.1 (Fig. 2B). Therefore, our data indicate that a large portion of the γδ T cells in the thymus of Id3−/− mice have an activated phenotype. The DN, CD4 and CD8 γδ T cells in the spleen of Id3−/− mice also have an activated phenotype (Fig. S3).

A subset of CD122+ γδ T cells, which are thought to have encountered ligand in the thymus, produce IFNγ rapidly after in vitro stimulation [43]. To determine whether Id3−/− γδ T cells represent previously activated cells we tested their ability to make IFNγ after in vitro stimulation with PMA and ionomycin for 5 hours. Importantly, >30% of Id3−/− TCRγ+ thymocytes produce IFNγ under these conditions. In contrast only 2.5% of Id3+/− TCRγ+ thymocytes produced IFNγ at this early time point (Fig 2C). Similarly, more than 50% of Id3−/− splenic TCRγ+ cells produced IFNγ (Fig S3). Interestingly, a subset of Id3−/− γδ T cells make both IFNγ and IL-4 (Fig. S4). Cytometric bead analysis revealed that Id3−/− γδ T cells also make more IFNγ, IL-4, IL-10 and IL-13 than their WT counterparts after stimulation with anti-TCR (Fig. S4). Taken together, our data demonstrate that Id3−/− mice develop a large population of γδ T cells that show characteristics of previously activated cells.

**Id3−/− γδ T Cells with an Activated Phenotype Develop Early in Post-Natal Life**

To determine when during ontogeny Id3−/− γδ T cell numbers increase and when the activated phenotype becomes evident, we examined thymocytes from mice isolated 1 week after birth. At this stage of ontogeny, few thymocytes have left the thymus and therefore peripheral activation is unlikely to have impacted on thymocyte numbers or phenotype. Importantly, a 10-fold increase in TCRγ+ cells was observed in Id3−/− neonates and the aberrant expression of CD4 was already evident (Fig. 3A, B and C). Moreover, Id3−/− neonatal γδ T cells had an activated phenotype similar to that observed in the adult Id3−/− thymus (Fig. 3D and E), although only a small subset of these cells were positive for NK1.1. Taken together, our data indicate that Id3 limits the development of γδ T cells with an activated phenotype.
in neonatal mice. Our data also indicate that the activated phenotype of Id3
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cd
T cells likely occurs within the thymus rather than as a consequence of peripheral activation since few thymocytes have left the thymus within the first week after birth [44].

The Majority of Id3
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cd
T Cells Express Vγ1.1 and Vδ6.3

The presence of a large population of activated γδ T cells in the Id3
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neonatal thymus suggests that these cells derive from cells that underwent V(D)J recombination in the late embryonic or neonatal period. To gain insight into the origin of the majority of Id3
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γδ T cells, we examined their TCR repertoire by staining with a panel of anti-Vγ antibodies. This analysis revealed that >90% of γδ T cells in the Id3
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thymus express Vγ1.1 (Fig. 4A, B). This increase in Vγ1.1 usage is not at the expense of the other Vγ gene segments since the total number of Vγ2 and Vγ5 γδ T cells are similar to that in the WT thymus, although their frequency within the γδ T cell population is reduced (Fig. 4C).

Importantly, the majority of Vγ1.1+ cells in the Id3
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thymus co-express Vδ6.3 (Fig. 4A). Vγ1.1†Vδ6.3+ γδ T cells have been reported to be of late fetal origin, express CD4 and have an activated phenotype including high expression of CD44, low expression of CD24 with rapid production of IFNγ and IL4 similar to what we have observed with Id3
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T cells [22]. These observations lead us to conclude that Id3 deficiency allows for an increase in the number of Vγ1.1†Vδ6.3+ γδ T cells without a major effect on Vγ2 or Vγ5 γδ T cells.
Limited Diversity in \(V_{\gamma 1.1-J}4\) and \(V_{\delta 6-J}\delta 1\)

Rearrangements in \(ld3^{-/-}\) \(\gamma\delta\) T Cells

In WT mice \(V_{\gamma 1.1-V}\delta 6.3^-\) \(\gamma\delta\) T cells develop from fetal precursors that rearrange the \(\gamma\) and the \(\delta\) chains in late embryonic life [23]. These cells show frequent rearrangement of the \(V_{\gamma 1.1}\) variable gene segment to the \(J_{\gamma 4}\) joining segment and of \(V_{\delta 6.3}\) to \(J_{\delta 1}\) and, depending on the genetic background of the mice, can have oligoclonal or polyclonal junctional sequences [45,46]. To gain insight into the complexity of the rearrangements in \(ld3^{-/-}\) \(V_{\gamma 1.1-V}\delta 6.3^-\) cells we amplified and sequenced the \(V_{\gamma 1.1-J}4\) and \(V_{\delta 6-J}\delta 1\) junctions in the TCR\(\gamma\delta\) population. Analysis of \(V_{\gamma 1.1-J}4\) junctions revealed that 30 of 31 sequences were in-frame and consisted of only two unique sequences indicating a population of \(V_{\gamma 1.1}\) T cells lacking significant TCR diversity. In addition, these sequences lacked \(N\) nucleotide additions suggesting that the rearrangements occurred in the absence of TdT (Fig. 5A). Analysis of \(V_{\delta 6-J}\delta 1\) junctions also revealed a lack of diversity with 32 of 37 in-frame sequences containing the \(V_{\delta 6.3}\) gene segment, consistent with our flow cytometry analysis (Fig. 5B). Moreover, 21 of the 32 \(V_{\delta 6.3-J}\delta 1\) junctions are represented by only two sequences. In the majority of sequences the \(D_{\delta 2-J}\delta 1\) and \(V_{\delta 6-D}\delta 2\) junctions resulted in maintenance of the germline sequence and the \(D_{\delta 1}\) gene segment was not observed in these junctions (Fig. 5B). Of the 4 unique sequences that showed diversity following the \(V_{\delta 6.3}\) gene segment at least 2 represent potential \(P\) rather than \(N\) nucleotide additions. Notably, the invariable
Dδ2-Jδ1 junction forces a unique reading frame of the Dδ segment (V/IGGIRA), which contributes to the CDR3 domain [47], thus resulting in a highly invariant Vγ1.1/Vδ6.3+ TCR, at least for those cells using the Vγ1.1-Jγ4 and Vδ6-Jδ1 rearrangement. The presence of a highly invariant receptor on cells with an activated phenotype suggests that the Vγ1.1+/Vδ6.3+ T cells are selected by a ligand present in the thymus.

To examine the possibility that the Vγ1.1+/Vδ6.3+ T cells in Id3−/− mice arise as a consequence of preferential Vγ1.1 and Vδ6.3 rearrangement in adult thymocytes we analyzed the Vγ1.1-Jγ4 and Vδ6-Jδ1 junctions in unselected Id3−/− DN3 cells. This analysis revealed that the in-frame Vγ1.1-Jγ4 rearrangements (8/15) contained 3 unique sequences that were distinct from those amplified from Id3+/− γδ T cells (Fig. 5A). In addition, only 3 of 16 Vδ6-Jδ1 junctions were in-frame and each of these sequences was unique with one sequence containing the Vδ6B gene segment. Therefore, Id3−/− DN3 cells show no evidence of a preferential production of the Vγ1.1-Jγ4 or Vδ6.3-Jδ1 junctions used in the γδ T cells in Id3−/− mice. Further, if a small number of Vγ1.1+/Vδ6.3+ γδ T cells with this rearrangement developed in the adult and expanded we would expect this population of γδ T cells to incorporate more BrdU than WT γδ T cells. However, multiple in vivo BrdU incorporation experiments failed to reveal an increase in proliferation of Id3−/− γδ T cells (Fig. S5) [35]. Taken together, our results indicate that in the absence of Id3 there is an elevated number of Vγ1.1+/Vδ6.3+ γδ T cells that
Id3 Limits Vγ1.1+Vδ6.3+ Cells

Figure 4. id3−/− γδ T cells are highly enriched for cells with a Vγ1.1/Vδ6.3 TCR. A) FACS analysis of thymocytes for TCRγδ and Vγ1.1 (top panel). The frequency of TCRγδ/Vγ1.1+ cells is shown. TCRγδ/Vγ1.1+ cells were analyzed for expression of Vδ6.3 (bottom panel). The frequency of Vγ1.1/Vδ6.3+ cells in the TCRγδ/Vγ1.1+ population is shown. B) Frequency of Vγ1.1+Vδ6.3− cells among TCRγδ+ cells in id3+/+ and id3−/− mice. Data were calculated by dividing the frequency of Vγ1.1+Vδ6.3− cells by the frequency of TCRγδ+ cells ×100. Each dot represents the frequency from one mouse. The line represents the average value. C) Analysis of thymocytes for expression of TCRγδ and Vγ2, Vγ5 or Vγ1 in id3+/+ and id3−/− thymocytes. One of 4 representative experiments is shown.

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Development of Activated γδ T Cells in id3−/− Mice Requires SAP

A subset of Vγ1.1+Vδ6.3+, referred to as γδ NKT, share phenotypic and functional characteristics with NKT cells including expression of the transcription factor PLZF and a requirement for SAP-dependent [24,37,39,48]. However, some Vγ1.1+Vδ6.3+ T cells develop independent of SAP signaling [24]. To further establish the parallels between id3−/− Vγ1.1+Vδ6.3+ cells and NKT cells we investigated the expression of PLZF. Importantly, PLZF was highly expressed in these cells compare to Vγ1.1+Vδ6.3+ γδ T cells, in both the id3+/+ and the id3−/− thymus (Fig. 6A). We examined whether development of this activated γδ T cell population in id3−/− mice requires SAP by generating id3−/−Sh2d1a−/− mice. Strikingly, the total number of γδ T cells in id3−/−Sh2d1a−/− mice was similar to that in WT and Sh2d1a−/− mice (Fig. 6B and C). Moreover, the frequency of Vγ1.1+Vδ6.3+ γδ T cells in id3−/−Sh2d1a−/− mice was similar to WT and id3−/−Sh2d1a−/− γδ T cells showed no evidence of an activated phenotype (Fig. 6D and Fig. S7). Therefore, activation of the SAP signaling pathway is essential for the γδ T cell phenotype observed in id3−/− mice. These data indicate that SAP is essential for development or survival of the Vγ1.1+Vδ6.3+ T cells present in id3−/− mice. Interestingly, all of the observed alterations in the id3−/− thymus were normalized by deletion of Sh2d1a. That is, γδ T cell numbers and phenotype as well as total thymocytes numbers are similar to WT in id3−/−Sh2d1a−/− mice (Fig. 6D). This finding is striking because deletion of γδ T cells in id3−/− mice, by creating id3−/−Tert−/− mice, does not restore thymic cellularity to WT levels (Fig. S8). Therefore, multiple alterations in the id3−/− thymus are dependent on SAP signaling.
Development of Activated γδ T Cells in Id3−/− Mice Requires E2A

Id3 is a transcriptional repressor that prevents E proteins from binding DNA [49]. All of the E proteins are expressed in T cells; however, deletion of E2A is sufficient to restore ab T cell maturation defects in Id3−/− [50]. Therefore, we tested the requirement for E2A in the development of activated γδ T cells in Id3−/− mice by generating Id3−/−E2A−/− mice. Consistent with a previous study we found that E2A is required for development of normal numbers of γδ T cells (Fig. 7A and B) [51]. Importantly, mice that lack both Id3 and E2A have fewer γδ T cells than WT mice but more γδ T cells than E2A−/− mice (Fig. 7A and B). Nonetheless, the γδ T cells that develop in Id3−/−E2A−/− mice fail to express CD122 and NK1.1 (Fig. 7C). Therefore, E2A is required for the development of γδ T cells with an activated phenotype in Id3−/− mice.

**Discussion**

In this study, we report that Id3-deficiency results in a 8-fold increase in the number of γδ T cells in the thymus and that the majority of these cells likely express an invariant Vγ1.1-Vδ6.3+ TCR. Similar to WT Vγ1.1-Vδ6.3+ cells, Id3−/− γδ T cells have high expression of CD122, CD44 and NK1.1, low expression of CD24, and rapidly secrete IFNγ and IL4 after in vitro stimulation. The “activated” phenotype of these γδ T cells parallels that of NKT cells, a finding that has led to the hypothesis that
Vγ1.1/Vδ6.3+ T cells represent an innate branch within the γδ T cell lineage [25]. Here, we demonstrate that both Id3+/+ and Id3−/− thymic Vγ1.1/Vδ6.3+ cells express the transcription factor PLZF, a molecular determinant of the NKT cell fate [37]. Moreover, we find that SAP is essential for development of Id3−/− Vγ1.1/Vδ6.3+ T cells, as is the case for NKT cells [39,52,53]. We, and others, have found that the majority of adult γδ T cells in Id3−/− mice proliferate at a rate similar to WT γδ T cells indicating that the Id3−/− Vγ1.1/Vδ6.3+ population is not increased because of extensive proliferation in the adult thymus [35], rather, we conclude that these γδ T cells expand during neonatal life. Our data are consistent with a model in which Id3 controls the response of Vγ1.1/Vδ6.3+ T cells to ligand- and/or SAP-mediated proliferation.

We demonstrate that the increased number of γδ T cells in Id3−/− mice is attributed to the increase in SAP-dependent cells. Deletion of Sh2d1a in Id3−/− mice abrogated the increase in γδ T cell numbers and the activated phenotype. Therefore, the major effect of Id3-deficiency on γδ T cell development is an increase in embryonically derived Vγ1.1/Vδ6.3+ T cells. This conclusion is in contrast to a previous report suggesting that alterations in adult DN3 cells underlie the increased production of γδ T cells in Id3−/− mice [53]. This conclusion was based, in part, on the observation that Id3−/− γδ T cells have less germline DNA at the TCRβ locus than WT γδ T cells. This finding led the authors to conclude that the γδ T cells developing in Id3−/− mice derive from cells that have an extended opportunity for TCRβ rearrangement. Our findings suggest that the reason for the increased TCRβ rearrangement may stem from differences in fetal versus adult cells rather than differences in Id3+/+ and Id3−/− adult DN3 cells. Our findings are also inconsistent with a model in which Id3 plays a critical role in selection of self-ligand reactive γδ T cells; however, many of the Id3−/− Vγ1.1/Vδ6.3+ cells express CD4 or CD8 which is consistent with a failure to prevent some aspects of γδ T cell development [54]. More importantly, our data reveal that SAP-dependent signaling pathways are critically linked to the altered phenotype of Id3−/− T cells since the thymus of Id3−/−/Sh2d1a−/− mice, unlike the Id3−/− or the Id3−/−/Tel−/− thymus, is indistinguishable from the Id3+/+ or Sh2d1a+/+ thymus with respect to cellularity and phenotype.

Our hypothesis that Id3 functions downstream of TCR signals to limit SAP-dependent proliferation in γδ T cells is consistent with previous studies demonstrating that Id3 is a target of TCR triggered signaling in both γδ and γδ T cells [30,32]. The pathway from the TCR leading to Id3 involves the MAP kinases Erk1 or Erk2, which are triggered by the Tec kinases Itk and Rlk [55].
Recently, Ik cycling mice were reported to have an increased number of PLZF-expressing Vc1.1+Vd6.3+ T cells, implying that Itk may also limit development of “innate” γδ T cells [25,56]. Our data are consistent with the hypothesis that Id3 is an essential effector of the TCR-Itk-MAP kinase pathway that determines the consequence of signaling through the Vc1.1+Vd6.3+ TCR. Id3 is an inhibitor of E protein DNA binding [57]. We, and others, found that deletion of E2A in Id3-/- mice blunted the development of activated γδ T cells indicating that elevated E2A activity is critical for development of these cells [35]. It should be noted that E2A is required for normal γδ T cells development and affects the timing of rearrangement of specific Vγ receptors [21,91]. Therefore, loss of activated γδ T cells in E2A-/- mice, as compared to Id3-/- mice, could be the result of E2A functions upstream of TCR signaling and independent of Id3. However, it seems likely that Id3 deletion leads to heightened E2A (or E protein) activity after TCR-initiated signaling events, where E2A activity would normally be inhibited. In the case of γδ T cells, elevated activity of E2A may cooperate with SAP-dependent signals to promote an outcome from TCR-mediated signaling that is not typical, for example, leading to prolonged survival or proliferation.

Our analysis of Vγ1.1-Jγ4 and Vδ6-Jδ1 sequences in Id3-/- γδ T cells and DN3 thymocytes lead us to conclude that the majority of Vγ1.1”Vδ6.3” cells in Id3-/- mice develop during fetal or neonatal life. This analysis revealed that Id3-/- γδ T cells have germline sequences at the Vc1.1-Jc4 and Dδ2-Jδ1 junctions, very low diversity in the Vδ6.3-Dδ2 junction and complete absence of the Dδ1 segment. However, Vγ1.1-Jγ4 and Vδ6-Jδ1 sequences retrieved from Id3-/- DN3 progenitors are characterized by diverse junctions. It is possible that the Vγ1.1-Jγ4 and Vδ6-Jδ1 sequences observed in Id3-/- γδ T cells could be generated from...
adult DN3 cells and that thymic selection leads to expansion of these cells. However, two observations argue against this possibility. First, Id3<sup>+/−</sup> γδ T cells proliferate to a similar extent as WT γδ T cells in the adult thymus and, second, development of Vγ1.1Vδ6.3<sup>+</sup> T cells is blunted in WT or Id3<sup>−/−</sup> mice reconstituted with Id3<sup>−/−</sup> adult bone marrow. Therefore, adult thymic progenitors do not efficiently recapitulate the γδ T cell phenotype observed in Id3<sup>−/−</sup> mice. The activated phenotype of Id3<sup>−/−</sup> γδ<sup>+</sup>Vγ1.1Vδ6.3<sup>+</sup> T cells is consistent with the hypothesis that this receptor recognizes a ligand in the thymus. γδ<sup>+</sup> T cells that recognize the unconventional MHC molecule T10- or T22 and Id3<sup>−/−</sup> mice, unless otherwise indicated. experiments were performed on mice that were 6 to 8 weeks old with a minimum of 2,000 TCR<sup>+</sup> cells. Since Vγ1.1Vδ6.3<sup>+</sup> T cells share many features with NKT cells including rapid production of IFNγ and IL-4, their increased numbers could significantly alter immune responses. Indeed, Id<sup>−/−</sup> mice, which have an increased number of Vγ1.1Vδ6.3<sup>+</sup> T cells, have elevated serum IgE that is dependent on γδ<sup>+</sup> T cells [25,56]. Therefore, while Id3 appears to be largely dispensable for development of conventional γδ<sup>+</sup> cells, it limits the number of PLZF-expressing SAP-dependent “inmate” γδ<sup>+</sup> T cells.

Materials and Methods

Ethics Statement

All animal experiments were performed in compliance with the requirements of the University of Chicago Institutional Animal Care and Use Committee.

Mice

Mice were housed at The University of Chicago Animal Resource Center. Id3<sup>−/−</sup> and Tcrd<sup>−/−</sup> mice were purchased from Jackson ImmunoResearch. S<sup>b2</sup>d<sup>1a</sup><sup>−/−</sup> mice were a kind gift from C. Terhorst. Genotyping was as previously described [59,60,61]. All experiments were performed on mice that were 6 to 8 weeks old unless otherwise indicated.

Antibodies, Flow Cytometry and Cell Sorting

Cells were blocked with anti-FcγR prior to staining with specific antibodies conjugated to biotin, FITC, PE, PE-Cy7 or APC, acquired in a FACS Canto using FACSDiva software and analyzed with FLOWjo. In all experiments viable cells were gated based on forward and side scatter profiles and dead cells were further excluded using Propidium iodide. Sorting was performed on a FACSAria. The following antibodies were purchased from Jackson ImmunoResearch: Flow Cytometry panel) or TCR<sup>+</sup>/CD8<sup>+</sup>/CD44/CD24/NK1.1. Data are representative of more than 10 independent experiments. C) Flow cytometric analysis showing intracellular IFNγ expression in Id3<sup>+/−</sup> and Id3<sup>−/−</sup>/TCRγδ<sup>+</sup> splenocytes 5 hours after stimulation with PMA and ionomycin. The shaded histogram shows staining with an isotype control antibody, open histogram shows staining with anti-IFNγ antibody. One of 3 independent experiments is shown. (D) CXβ<sup>−/−</sup>/CD8α<sup>+</sup> splenocytes analyzed for expression of TCRβ (left panel) or TCRγδ (right panel) and CDββ. (E). Flow cytometric analysis of CXβ<sup>−/−</sup>/CD8α<sup>+</sup> splenocytes analyzed for expression of TCRβ (left panel) or TCRγδ (right panel) and CDββ.

Figure S4 Id3<sup>−/−</sup> γδ<sup>+</sup> T cells make IFNγ and IL-4 after in vitro stimulation. (A) Total thymocytes from Id3<sup>−/−</sup> or Id3<sup>+/−</sup> mice were cultured in vitro with [lower panels] or without [upper panels] PMA plus ionomycin for 5 hours. Intracellular staining for IFNγ and IL-4 on TCRγδ<sup>+</sup> cells is shown. The frequency of cells producing both IFNγ and IL-4 is indicated. (B) Cytometric bead assay for IFNγ, IL-4, IL-10 and IL-13 produced from anti-CD19, anti-TCRβ and anti-Ter19 depleted splenocytes 72 hours after stimulation with anti-TCRδ antibody. PMA+ionomycin stimulated thymocytes from Id3<sup>−/−</sup> mice are shown as a positive control.

Supporting Information

Figure S1 Id3<sup>−/−</sup> mice have 3-fold fewer thymocytes than Id3<sup>+/−</sup> mice. Total number of thymocytes in Id3<sup>+/−</sup> and Id3<sup>−/−</sup> mice. Bars represent the average ± standard deviation from at least 10 mice. p<0.0005. Found at: doi:10.1371/journal.pone.0009303.s001 (2.54 MB TIF)

Figure S2 Id3<sup>−/−</sup> mice have an increased number of γδ<sup>+</sup> T cells in the spleen that express CD4 and CD8. A) Flow cytometric analysis of Id3<sup>+/−</sup> and Id3<sup>−/−</sup> splenocytes for TCRβ and TCRγδ. Total splenocytes were first gated for viable cells using propidium iodide (PI). B) Total number of TCRγδ<sup>+</sup> cells in the spleen of Id3<sup>+/−</sup> and Id3<sup>−/−</sup> mice. Bars represent the average ± standard deviation from >15 mice. p<0.001. C) TCRγδ<sup>+</sup> cells were analyzed for CD4 and CD8 expression. D) Total number of CD4<sup>+</sup>, CD8<sup>+</sup>, DN and DP splenocytes expressing TCRγδ in the spleen Id3<sup>+/−</sup> (grey) and Id3<sup>−/−</sup> (black) mice. Bars represent the average ± standard deviation from >15 mice. p<0.001 for all Id3<sup>−/−</sup> to Id3<sup>+/−</sup> comparisons. E) Flow cytometric analysis of Id3<sup>−/−</sup>/CD8α<sup>+</sup> splenocytes analyzed for expression of TCRβ (left panel) or TCRγδ (right panel) and CDββ.

Figure S3 Id3<sup>−/−</sup> γδ<sup>+</sup> T cells have characteristics of activated cells. Flow cytometric analysis of Id3<sup>+/−</sup> DN splenocytes (A) or Id3<sup>−/−</sup> DN, CD4 or CD8 splenocytes (B) for expression of CD122, CD44, CD24 or NK1.1. Data are representative of more than 10 independent experiments. C) Flow cytometric analysis showing intracellular IFNγ expression in Id3<sup>+/−</sup> and Id3<sup>−/−</sup>/TCRγδ<sup>+</sup> splenocytes 5 hours after stimulation with PMA and ionomycin. The shaded histogram shows staining with an isotype control antibody, open histogram shows staining with anti-IFNγ antibody. One of 3 independent experiments is shown. Found at: doi:10.1371/journal.pone.0009303.s002 (10.44 MB TIF)
were injected into lethally irradiated (1000 rad) mice and thymocytes from these mice were analyzed 6 or 12 weeks post-reconstitution. Flow cytometric analysis for TCRβ and TCRγδ on total thymocytes is shown. Plots are representative of 2–3 independent experiments. A) Total thymocytes numbers in mice of the indicated genotypes. At least 4 mice were analyzed for each genotype. 

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