Differential Requirement of RasGRP1 for γδ T Cell Development and Activation

Yong Chen, Xinxin Ci, Balachandra Gorentla, Sarah A. Sullivan, James C. Stone, Weiguo Zhang, Pablo Pereira, Jianxin Lu and Xiao-Ping Zhong

J Immunol 2012; 189:61-71; Prepublished online 23 May 2012; doi: 10.4049/jimmunol.1103272
http://www.jimmunol.org/content/189/1/61
The Journal of Immunology

Differential Requirement of RasGRP1 for γδ T Cell Development and Activation

Yong Chen,*† Xinxin Ci,*† Balachandra Gorentla,* Sarah A. Sullivan,§ James C. Stone,*† Weiguo Zhang,§ Pablo Pereira,# Jianxin Lu,† and Xiao-Ping Zhong*§

γδ T (γδT) cells belong to a distinct T cell lineage that performs immune functions different from αβ T (αβT) cells. Previous studies established that Erk1/2 MAPKs are critical for positive selection of αβT cells. Additional evidence suggests that increased Erk1/2 activity promotes γδT cell generation. RasGRP1, a guanine nucleotide-releasing factor for Ras, plays an important role in positive selection of αβT cells by activating the Ras–Erk1/2 pathway. In this article, we demonstrate that RasGRP1 is critical for TCR-induced Erk1/2 activation in γδT cells, but it exerts different roles for γδT cell generation and activation. Deficiency of RasGRP1 does not obviously affect γδT cell numbers in the thymus, but it leads to increased γδT cells, particularly CD4⁺CD8⁻ γδT cells, in the peripheral lymphoid organs. The virtually unhindered γδT cell development in the RasGRP1⁻/⁻ thymus proved to be cell intrinsic, whereas the increase in CD8⁺ γδT cells is caused by non–cell-intrinsic mechanisms. Our data provide genetic evidence that decreased Erk1/2 activation in the absence of RasGRP1 is compatible with γδT cell generation. Although RasGRP1 is dispensable for γδT cell generation, RasGRP1-deficient γδT cells are defective in proliferation following TCR stimulation. Additionally, RasGRP1-deficient γδT cells are impaired to produce IL-17 but not IFNγ. Together, these observations revealed that RasGRP1 plays differential roles for γδ and αβ T cell development but is critical for γδT cell proliferation and production of IL-17. The Journal of Immunology, 2012, 189: 61–71.

Two lineages of T cells marked by the expression of two distinct Ag receptors, αβ and γδ TCRs, are generated during intrathymic development. T cell development in the thymus can be divided into CD4⁺CD8⁻ (double-negative [DN]), CD4⁺CD8⁺ (double-positive [DP]), and CD4⁻CD8⁺ or CD4⁻CD8⁻ (single-positive [SP]) stages. DN thymocytes contain the most immature T cells and can be divided into DN1 to DN4 stages based on CD25 and CD44 or cKit expression (1). Functional TCRs must be generated through somatic V(D)J recombination in the TCR loci for generation of either αβ T (αβT) cells or γδ T (γδT) cells (2). V(D)J recombination in the TCR loci is tightly regulated in a developmental stage-specific manner. At the DN2 and DN3 stages, TCRγ, δ, and β loci rearrange. Formation of functional TCRγδ directs progenitor cells to the γδ lineage (3). TCRβ associates with the pre-TCR-αβ chain to form the pre-TCR, which drives DN thymocyte maturation to the DP stage and full commitment to the αβT cell lineage (4). DN2 thymocytes are mostly committed to the T cell lineage; γδT lineage commitment mainly occurs at the DN2 stage, but it can also happen at the DN3 stage (5). At the DP stage, the TCRs gene rearranges, and in-frame rearranged α gene produces a functional chain to associate with the TCRβ-chain to drive DP thymocytes to mature to the SP stage (6). In normal thymus and peripheral lymphoid organs, γδT is the minor lineage, whereas αβT is the dominant lineage. Most γδT cells reside in the DN population, and γδT cells expressing CD4 or CD8 coreceptor are rare.

It has been well documented that expression of a functional TCRγδ or TCRαβ in developing thymocytes is essential for the generation of their respective T cell lineages. Defects in the formation of a functional TCRγδ or TCRαβ can cause a complete absence of the γδ or αβ T cell lineage, respectively (3, 7, 8). Our knowledge of TCR signal transduction has primarily come from studies of TCRβ, because γδT cells are rare. It is well known that TCR stimulation leads to the activation of phospholipase Cγ1 via orchestrated actions of proximal protein kinases, such as Lck, Zap70, and Itk, and adaptor molecules, such as SLP-76 and LAT (9–12). Activated phospholipase Cγ1 produces two critical second messengers, diacylglycerol and inositol-1,4,5-trisphosphate, which are crucial for relaying proximal signaling to the activation of distal signaling cascades (13). Inositol-1,4,5-trisphosphate binds to its receptor in the endoplasmic reticulum, leading to depletion of calcium from the endoplasmic reticulum and subsequent calcium influx through the calcium release-activated calcium channel, which leads to the activation of the calcineurin–NFAT pathway (14). Diacylglycerol associates with multiple effector molecules, including RasGRP1, PKCθ, and PKDs, to induce the activation of

© 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00

Received for publication November 15, 2011. Accepted for publication April 22, 2012.

This work was supported by funding from the National Institutes of Health (R01AI076357, R01AI079088, and R21AI079873), the American Cancer Society (RSG-08-186-01-LIB to X.-P.Z.), and the Chinese National Science Foundation (31071237).

Address correspondence and reprint requests to Dr. Xiao-Ping Zhong or Dr. Jianxin Lu, Division of Allergy and Immunology, Department of Pediatrics, Room 133, MSRB-I, Research Drive, Box 2644, Duke University Medical Center, Durham, NC 27710 (X.-P.Z.) or Key Laboratory of Laboratory Medicine, Ministry of Education, School of Laboratory Medicine, Wenzhou Medical University, Wenzhou, Zhejiang Province 325035, China (J.L.). E-mail addresses: zhong001@mc.duke.edu (X.-P.Z.) and jxl813@163.com (J.L.)

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; BM, bone marrow; DN, double negative; DP, double positive; KO, knockout; LN, lymph node; mTOR, mammalian target of rapamycin; SP, single positive; WT, wild-type.
downstream signaling cascades, such as the RasGRP1–Ras–Erk1/2 and PKCθ–CARMA1/Blc10–IKK–NF-κB pathways (13, 15, 16).

Evidence suggests that TCRβ and TCRγδ signaling share at least some common features. Deficiency of some proximal signaling molecules, such as Lck, SLP76, and LAT, impacts both αβT cell and γδT cell generation (17). Additionally, expression of a TCRβ at early DN stages in transgenic mice can drive thymocytes to adopt the γδ fate, even though they express TCRβ, suggesting that the timing of TCRγδ versus TCRβ signaling, rather than the quality of the signaling, plays an important role in γδ versus αβ lineage fate decision (18). Although similarities between αβ and γδ TCRs exist, differences between them have also been reported. Murine TCRβ, but not TCRγδ, contains the CD3δ chain (19, 20). The threshold of TCRγδ signaling appears lower than for TCRβ. Under the same stimulating condition, TCRγδ induces stronger Erk1/2 activation than does TCRβ. Furthermore, strong TCR signaling and Erk1/2 activation were reported to promote γδ differentiation (19, 21–24). However, substantial genetic evidence demonstrating the requirement of Erk1/2 for γδT cell lineage development is lacking.

RasGRP1, a guanine nucleotide exchange factor for Ras, is critical for the activation of the Ras–Erk1/2 pathway in αβT cells (25, 26). RasGRP1 promotes positive selection of conventional αβT cells, particularly those expressing TCR with low affinity to the self-peptide–MHC complex (27). We recently found that RasGRP1 also promotes the development of the invariant NKT cells (28). However, positive selection of thymocytes with relatively high affinity to the self-peptide–MHC complex, such as regulatory T cells, is less dependent on RasGRP1 (29). Although the importance of RasGRP1 in αβT cells is becoming clear, whether RasGRP1 functions as the upstream activator for Erk1/2 during TCRγδ signaling and its importance for γδT development and activation are not well understood. In this article, we demonstrate that RasGRP1 is important for TCR-induced Erk1/2 activation in γδT cells but is dispensable for γδT cell generation. Deficiency of RasGRP1 does not obviously affect γδT cell numbers in the thymus but leads to increased γδT cells, particularly CD4−CD8− γδT cells, in the peripheral lymphoid organs. Although RasGRP1 is not required for γδT cell ontology, it is critical for TCR-induced γδT cell proliferation and production of IL-17.

Materials and Methods

Mice

C57BL/6J mice were purchased from The Jackson Laboratory. RasGRP1−/− mice were described previously (25) and were backcrossed onto the B6 background for nine generations. All mice used were between 6 and 8 wk of age, according to a protocol approved by the Duke University Institute Animal Care and Use Committee. Thymocytes, splenocytes, and lymph node (LN) cells were prepared following standard procedures.

Abs and flow cytometry

Cells were stained with Abs in PBS containing 2% FCS. PE-Cy7–conjugated anti-CD3 (eBioscience) after cell surface staining, followed by allophycocyanin−PE- or allophycocyanin-conjugated anti-CD90.2 (30-H12) were purified to isolate αβT cells, as previously described (31). Cells were stained for death with Live/Dead fixable staining before being analyzed by flow cytometry.

For cytokine production, 2×10^6 freshly isolated thymocytes, splenocytes, and LN cells were seeded in each well of a 48-well plate precoated with PBS or with 10 μg/ml soluble anti-CD3ε (2C11). After incubation at 37°C for 72 h, cells were stained for death with Live/Dead fixable staining before being analyzed by flow cytometry.

To induce γδT cell development, 4×10^6 αβT cells were labeled with 10^5 ID3; for-ward, 5’-GTCGTTTTCTCAGATGGCTCTGA-3’ and reverse 5’-CA-GTGTTTGTGCTGGGATA-3’; Egr2: forward, 5’-TTGGACAGATGGAC-GGAGTG-3’ and reverse 5’-CAGATGGAGGAAGCACTG-3’; Il3: forward, 5’-CCTAGACGGCTGACCTGAGCC-3’ and reverse 5’-CCAAAAGGCCG-3’. Relative mRNA expression was normalized with β-actin and was presented as an arbitrary unit of fold change using the 2^−ΔΔCT method.

Statistics

For statistical analysis, two-tailed Student t tests were performed.

Results

RasGRP1 is dispensable for intrathymic γδT cell development

We first examined mRNA levels of RasGRP1 and other RasGRP family members in γδT cells because it has been unclear whether these molecules are expressed in these cells. RasGRP1, 2, and 4 mRNAs could be detected in both γδT cells and αβT cells sorted from the thymus and spleens (Fig. 1A, 1B). αβT cells appeared to

Bone marrow reconstitution

Wild-type (WT) C57BL/6J mice were lethally irradiated (1100 rad) 4 h before adoptive transfer. Bone marrow (BM) cells from age- and sex-matched Thy1.1 B6 and Thy1.2 RasGRP1−/− mice were mixed at a 1:1 ratio. Ten to twenty million mixed cells were then injected i.v. into each recipient mouse. The resulting chimeric mice were analyzed 7–8 wk later.

Western blot

Total γδT cells were isolated from WT and RasGRP1−/− thymocytes, splenocytes, and LN cells using MACS purification and then FACS sorting using a MoFlo Cell Sorter (Beckman Coulter), with postsort purity >98%. Thymic and splenic γδT cells were also similarly purified from these mice. A total of 400,000 sorted γδT cells was rest in 0.5 ml PBS at 37°C for 30 min. Cells were then either left untreated or stimulated with an anti-CD3ε Ab (500A2, 5 μg/ml; BD Biosciences) for 5 min. The 500A2 Ab is capable of inducing TCR signaling without cross-linking by a secondary Ab. Cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris [pH 7.4]) with protease and phosphatase inhibitors. Proteins in lysates were subjected to Western blot analysis with anti-phospho-Erk1/2, total Erk1/2 Abs (Cell Signaling Technology), and an anti-RasGRP1 Ab (Santa Cruz Biotechnology). For loading control, the blots were stripped and reprobed with anti-β-actin (Sigma).

γδ T cell proliferation and cytokine production

Sorted WT and RasGRP1−/− γδT cells were labeled with 10 μM CFSE at room temperature for 9 min, as previously described (31). Cells were seeded at 5×10^5 cells/well in a 48-well plate precoated with PBS or with 10 μg/ml soluble anti-CD3ε (2C11). After incubation at 37°C for 72 h, cells were stained for death with Live/Dead fixable staining before being analyzed by flow cytometry.

For cytokine production, 2×10^5 freshly isolated thymocytes, splenocytes, and LN cells were seeded in each well of a 48-well plate. The cells were left unstimulated or were stimulated with PMA (25 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug for 5 h. Cells were then surface stained for TCRγδ, TCRβ, CD4, CD8, CD27, and CD44, followed by intracellular staining for IFNy and IL-17A.

Real-time PCR

Total RNAs were extracted from sorted cells, and cDNAs were obtained using the Superscript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using RealMasterMix and performed on the Mastercycler ep realplex2 system (both from Eppendorf). Primers for RasGRPs and actin were published previously (25) or are as follows: Egr1: forward, 5’-GTCGTTTTCTCAGATGCTCTGA-3’ and reverse 5’-CA-GTGTTTGTGCTGGGATA-3’; Egr2: forward, 5’-TTGGACAGATGGAC-GGAGTG-3’ and reverse 5’-CAGATGGAGGAAGCACTG-3’; Il3: forward, 5’-CTAGACGGCTGACCTGAGCC-3’ and reverse 5’-CCAAAAGGCCG-3’. Relative mRNA expression was normalized with β-actin and was presented as an arbitrary unit of fold change using the 2^−ΔΔCT method.

Statistics

For statistical analysis, two-tailed Student t tests were performed.
express higher levels of RasGRP1 and RasGRP2 than did γδT cells. The differences were particularly obvious in the peripheral lymphoid organs. In contrast, expression of RasGRP4 was similar between αβT and γδT cells. RasGRP3 was undetectable in either γδT or αβT cells.

To determine the role of RasGRP1 in γδT cells, we analyzed RasGRP1−/− mice. As previously reported (21, 24), CD4⁺CD8⁻ and CD4⁺CD8⁺ SP thymocytes were markedly decreased in RasGRP1−/− (knockout [KO]) mice compared with WT mice (Fig. 1C, 1D). In addition, we found that the CD4⁺CD8⁺ DP thymocyte number, as well as total thymic cellularity, of RasGRP1−/− mice was decreased ∼50% (Fig. 1D). Further analysis of DN thymocytes revealed an increase in CD25⁺CD44⁻ DN3 cells and a decrease in CD25⁺CD44⁺ DN4 cells in RasGRP1−/− mice (Fig. 1E). These data suggested that RasGRP1 deficiency resulted in a partial blockage of the DN3 to DN4 transition, a process called β selection, because of its requirement of the pre-TCR signal. Thus, in addition to promoting positive selection, RasGRP1 may be involved in pre-TCR signaling and plays a role in β-selection.

In contrast to the striking decrease in αβT cells and DP thymocytes, the overall percentage of γδT cells in the RasGRP1−/− thymus was increased ∼2-fold compared with WT controls (Fig. 1F, 1G). Although rare, the relative ratio of γδT cells in the CD4SP or CD8SP population was also increased. The total thymic and DN

**FIGURE 1.** RasGRP1 is dispensable for γδ T cell development in the thymus. Expression of RasGRPs in αβT and γδT cells. mRNA levels of RasGRPs from sorted αβT and γδT cells from thymus (A) and spleen (B) were determined by real-time quantitative RT-PCR. (C) CD4 and CD8 staining of total thymocytes from WT and RasGRP1−/− mice. (D) Decrease in DP and SP thymocytes in RasGRP1−/− mice. Data shown are mean ± SEM. (E) Inefficient DN3 to DN4 transition in the absence of RasGRP1−/−. Representative FACS plots of CD25 and CD44 expression in gated CD4⁺CD8⁻ DN thymocytes are shown. (F) Representative FACS plots of TCRγδ and TCRβ staining of the indicated thymocytes. Total thymocytes, as well as gated DN and SP cells, are shown. (G) Percentages (left panel) and absolute number (right panel) of γδT cells within total, CD4SP, CD8SP, and DN thymocytes in WT and RasGRP1−/− mice. Mice used in the study were 6–8 wk of age. Data shown are representative of at least three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test.
and CD8⁺ γδT cell subsets in RasGRP1⁻/⁻ mice were similar to WT mice. However, the CD4⁺ γδT cell number was decreased slightly in RasGRP1⁻/⁻ mice. Thus, although RasGRP1 is critical for DP to SP maturation and is important for efficient DN3 to DN4 transition, it is virtually dispensable for intrathymic γδT cell development.

**Enrichment of γδT in the peripheral lymphoid organs in RasGRP1⁻/⁻ mice**

As previously reported (25, 27), CD4⁺ and CD8⁺ T cell percentages and numbers in the spleen and LNs were decreased in RasGRP1⁻/⁻ mice compared with WT mice (Fig. 2A). Different from the decrease in αβT cells, the percentages and absolute numbers of overall γδT cells did not decrease; in fact, they even increased in the spleen (Fig. 2B, 2D) and LNs (Fig. 2C, 2E) of RasGRP1⁻/⁻ mice. The percentages of splenic total and DN γδT cells were slightly decreased in the spleen but were more substantially increased in the LNs in RasGRP1⁻/⁻ mice. The total and DN γδT cell numbers in the WT and RasGRP1⁻/⁻ spleens were similar but were increased 2–4-fold in RasGRP1⁻/⁻ LNs. The reason for the selective increase in γδT cells in the LNs but not in the spleen is unclear, but it could be caused by altered expression of chemokine receptors or homing molecules, such as CD62L, in the absence of RasGRP1. The total CD4SP γδT cells were slightly decreased in both RasGRP1⁻/⁻ spleens and LNs. However, the percentages and absolute numbers of CD8SP γδT cells were drastically increased in both RasGRP1⁻/⁻ spleens and LNs. Although CD8αSP γδT cells accounted for ~0.5% of CD8SP T cells in WT mice, up to 40% of LN and 20% of splenic CD8SP cells from RasGRP1⁻/⁻ mice were γδT cells. About 90% of CD8SP γδT cells in WT and RasGRP1⁻/⁻ thymus and LN were CD8α⁺β⁻. In the spleen, CD8α⁻β⁺ cells accounted for 65% and 85% of WT and RasGRP1⁻/⁻ CD8SP γδT cells, respectively (Fig. 2F).

**Cell-intrinsic and -extrinsic mechanisms control γδ T cell development in RasGRP1⁻/⁻ mice**

Because the RasGRP1⁻/⁻ mice that we studied were germ-line KO and the overall T cell lymphopenia could significantly affect γδT cell development, we investigated whether γδT cell development in RasGRP1⁻/⁻ mice was caused by cell-intrinsic mechanisms. We generated and analyzed mixed BM chimeric mice reconstituted with Thy1.1⁺ WT and Thy1.2⁺ RasGRP1⁻/⁻ BM cells at a 1:1 ratio. Chimeric mice were analyzed 8 wk after transfer. The ratio of Thy1.1⁺ WT/Thy1.2⁺ RasGRP1⁻/⁻ CD4⁻ CD8⁻ DN thymocytes was ~2:1 (Fig. 3A), suggesting that RasGRP1 may be involved in early T cell development. Within the DN population, Thy1.2⁺ RasGRP1⁻/⁻ CD25⁺/CD44⁻ DN3 and CD25⁺/CD44⁺ DN4 cells were substantially increased and decreased, respectively, as compared with Thy1.1⁺ WT controls (Fig. 3B), indicating that RasGRP1 is intrinsically important for efficient β selection. These observations, together with those demonstrating critical roles for RasGRP1 in DP to SP maturation (25), indicate that RasGRP1 plays crucial roles at multiple stages of αβT cell development in a cell-autonomous manner. Within TCRαβ⁺ T cells, most were Thy1.1⁺ WT; Thy1.2⁺ RasGRP1⁻/⁻ cells were virtually undetectable (Fig. 3C). However, the ratio of γδT cells originated from RasGRP1⁻/⁻ to WT BM was similar to the ratio of total DN cells between these two origins. As mentioned earlier, CD8⁺ γδT cells were drastically increased in RasGRP1⁻/⁻ mice, but such an increase was not observed in the chimeric mice (Fig. 3D). Thus, the overall RasGRP1-independent generation of γδT cells in RasGRP1⁻/⁻ mice was cell intrinsic; however, the expansion of CD8⁺ γδT cells in these mice was not.

**Differential effects of RasGRP1 deficiency on αβT and γδT cell homeostasis**

The TCR-mediated Ras–Erk1/2 pathway plays a crucial role in T cell survival and proliferation. One possibility that may cause increased γδT cells in RasGRP1⁻/⁻ mice is that they have proliferative and/or survival advantage over αβT cells. Previous studies demonstrated that RasGRP1 prevents conventional CD4⁺ T cells and CD8⁺ T cells, as well as invariant NKT cells, from apoptosis (28, 29). By staining freshly isolated cells with Annexin V and 7AAD, we also observed increased apoptosis of RasGRP1⁻/⁻ αβT cells. However, no increase in apoptosis was observed in RasGRP1⁻/⁻ γδT cells compared with WT controls. In fact, slight decreases in apoptosis were observed in γδT cells from RasGRP1⁻/⁻ spleen and LNs (Fig. 4A).

In RasGRP1⁻/⁻ mice, the total peripheral αβT cell numbers are decreased. Under such a lymphopenic condition, T cells may undergo homeostatic proliferation. Expression of nuclear Ki67, a protein that is upregulated in actively cycling cells, was obviously increased in freshly isolated RasGRP1⁻/⁻ αβT cells compared with WT controls (Fig. 4B). Consistent with increased Ki67 expression in RasGRP1⁻/⁻ αβT cells, most of these cells were CD44⁺/CD62L⁻, which was consistent with homeostatic proliferation of these cells (Fig. 4C). In contrast, Ki67 expression in RasGRP1⁻/⁻ γδT cells was similar to WT controls (Fig. 4B). Furthermore, most RasGRP1⁻/⁻ γδT cells displayed a naive CD44⁺/CD62L⁺ phenotype (Fig. 3D). Of note, Ki67⁺ CD4SP RasGRP1⁻/⁻ γδT cells were increased compared with WT controls (Fig. 4B), which was likely caused by homeostatic proliferation due to the decrease of this population of γδT cells in RasGRP1⁻/⁻ mice.

Together, these observations suggest that RasGRP1⁻/⁻ αβT cells undergo enhanced homeostatic proliferation in vivo and that there is no obvious increase in homeostatic proliferation of most γδT cells in RasGRP1⁻/⁻ mice, with the exception of the CD4SP subset. Our data also suggest that γδT cells may sense different homeostatic cues compared with αβT cells, because the overall αβT cell lymphopenia resulted in increased homeostatic proliferation of αβT, but not γδT, cells in RasGRP1⁻/⁻ mice. The enrichment of γδT cells in the absence of RasGRP1 is likely an active process involved in γδT cell generation, because the percentage of intracellular TCRβ⁺ γδT cells was significantly decreased in RasGRP1⁻/⁻ mice (Fig. 4E).

**Requirement of RasGRP1 for TCR-induced Erk1/2 activation in γδT cells**

As shown in Fig. 1A, in addition to RasGRP1, RasGRP2 and 4 are expressed in γδT cells. We examined whether RasGRP1 deficiency may affect RasGRP2 and RasGRAP4 expression. As shown in Fig. 5A, the RasGRP2 mRNA level decreased ~50%, whereas the RasGRAP4 mRNA level increased ~20-fold in RasGRP1 KO γδT cells compared with WT controls. Increased Erk1/2 activity was found to promote γδT cell development (21, 23). It is also known that RasGRP1 plays an important role in TCR-induced Erk1/2 activation in total thymocytes and αβT cells (25, 27). The minimal requirement of RasGRP1 for γδT cell development and the drastic increase in RasGRAP4 expression in RasGRP1 KO γδT cells raise the possibility that RasGRP1 is dispensable for TCR-induced Erk1/2 activation or that increased RasGRAP4 expression compensates for the loss of RasGRP1 in TCR-induced Erk1/2 activation in γδT cells. To determine the role of RasGRP1 in TCR-induced Erk1/2 activation, we sorted γδT cells from RasGRP1⁻/⁻ and WT mice. Sorted cells were left unstimulated or were stimulated with a soluble anti-CD3 Ab, and Erk1/2 phosphorylation was examined by Western blot analysis. As shown in
Fig. 5B, Erk1/2 phosphorylation was decreased in RasGRP1<sup>−/−</sup> γδT cells. However, induction of Erk1/2 phosphorylation could still be detected in RasGRP1<sup>−/−</sup> γδT cells. Thus, RasGRP1 is required for optimal TCR-induced Erk1/2 activation in γδT cells, and other Ras activation mechanisms are not able to fully compensate for the loss of RasGRP1 in TCR-induced Erk1/2 activation.

Activation of the Ras–Erk1/2 pathway leads to induction of Egr1-3, which promotes Id3 transcription. Both Id3 and Egr1/2 are
involved in γδT cell development. Id3-deficient mice contain increased γδT cells that are dominantly Vγ1*Vδ6.3* innate γδT cells, which display an activating phenotype (32–36). Egr1/2 and Id3 expression was substantially decreased in RasGRP1+/− γδT cells (Fig. 5C). The decreased expression of Id3 cells may contribute to the ∼2-fold increase in Vγ1*Vδ6.3+ γδT cells in RasGRP1+/− mice (Fig. 5D, 5E).

Requirement of RasGRP1 for TCR-induced γδT cell proliferation

RasGRP1 activates the Ras–Erk1/2 pathway in peripheral T cells and is critical for peripheral αβT cell activation (25–27). The dispensable role for RasGRP1 in γδT cell development and homeostasis raises the question of whether this protein participates in γδT cell activation. Because of downregulation of TCRγδ following TCR activation, we sorted γδT cells and labeled them with CFSE. CFSE-labeled γδT cells were left unstimulated or were stimulated with plate-bound anti-CD3 for 72 h. Although WT γδT cells proliferated vigorously, RasGRP1+/− γδT cells were defective in proliferation (Fig. 6A). No obvious difference in cell death was observed between WT and RasGRP1 KO γδT cells during in vitro stimulation (Fig. 6B), suggesting that defective γδT cell proliferation in the absence of RasGRP1 was not caused by increased cell death. In WT mice, anti-CD3ε-induced Erk1/2 phosphorylation in splenic γδT cells was stronger than in thymic γδT cells. Both thymic and splenic RasGRP1 KO γδT cells displayed decreased Erk1/2 phosphorylation compared with WT controls (Fig. 6C). Given the role of the Ras–Erk1/2 pathway for αβT cell activation, impaired Erk1/2 activation in RasGRP1 KO γδT cells may lead to their proliferative defect. RasGRP1 KO γδT cells expressed slightly increased CD25 and CD122 compared with WT controls. They expressed similar levels of costimulatory molecules ICOS, CD5, OX40, and NKG2D compared with WT controls (Fig. 6D), suggesting that RasGRP1 is not required for the expression of these molecules. However, it is unclear whether RasGPR1 is involved in costimulatory signaling in γδT cells. Together, these observations indicate that RasGRP1 is crucial for TCR-induced γδT cell proliferation.

Defective IL-17 production by γδT cells in the absence of RasGRP1

γδT cells are an important innate source of IL-17 and IFN-γ (37–39). To examine whether RasGRP1 deficiency affects cytokine production by γδT cells, we stimulated freshly isolated thymocytes, splenocytes, and LN cells with PMA and ionomycin for 5 h in the presence of GolgiPlug, and we analyzed cytokine production by intracellular staining. IL-17A production by RasGRP1+/− γδT cells from the thymus, spleen, and LN was substantially...
decreased (Fig. 7A). In contrast to IL-17, IFN-γ production was increased in RasGRP1<sup>−/−</sup> gd<sup>T</sup> cells compared with WT gd<sup>T</sup> cells. Further comparison of cytokine expression in CD4<sup>+</sup>, CD8<sup>+</sup>, and DN gd<sup>T</sup> cells revealed that CD8<sup>+</sup> gd<sup>T</sup> cells produced higher levels of IFN-γ, but lower levels of IL-17A, than did CD4<sup>+</sup> and DN gd<sup>T</sup> cells in WT mice. In the absence of RasGRP1, IFN-γ expression was increased, but IL-17A expression was substantially decreased, in these gd<sup>T</sup> cell populations (Fig. 7B).

gd<sup>T</sup> cells can be divided into functional subsets based on CD27 and CD44 expression, with the CD27<sup>−</sup>CD44<sup>+</sup> subset mainly producing IL-17 and the CD27<sup>+</sup>CD44<sup>−</sup> subset producing IFN-γ (40). In RasGRP1 KO thymus and spleen, the percentages of CD27<sup>−</sup>CD44<sup>+</sup> gd<sup>T</sup> cells were decreased ~50 and 30%, respectively, compared with WT controls, suggesting that RasGRP1 is important for the generation/maintenance of this population of gd<sup>T</sup> cells (Fig. 7C). Moreover, IL-17 production by RasGRP1 KO CD27<sup>−</sup>CD44<sup>+</sup> gd<sup>T</sup> cells was substantially decreased compared with WT controls. In contrast to CD27<sup>−</sup>CD44<sup>+</sup> gd<sup>T</sup> cells, there were ~50–70% increases in CD27<sup>+</sup>CD44<sup>−</sup> gd<sup>T</sup> cells in RasGRP1 KO thymus and spleen. Moreover, IFN-γ production by splenic and LN CD27<sup>+</sup>CD44<sup>−</sup> gd<sup>T</sup> cells from RasGRP1 KO mice appeared to increase (Fig. 7D). Together, these observations revealed that RasGRP1 is not only important for the generation/maintenance of the CD27<sup>−</sup>CD44<sup>+</sup> gd<sup>T</sup> cell subset but is also critical for this subset of cells to produce IL-17.

Discussion

The activation of Erk1/2 is an important signaling event following both TCRβ and TCRγδ stimulation. In αβ<sup>T</sup> cells, RasGRP1 is a critical upstream activator for Erk1/2 through the Ras–Raf–Mek1/2 signaling cascade following TCR engagement (27). Genetic evidence revealed that both RasGRP1 and Erk1/2 are critical for maturation of T cells adopting the αβ<sup>T</sup> cell lineage, including conventional αβ<sup>T</sup> cells (25, 27, 41), invariant NKT cells (28),...
and regulatory T cells (29). In this article, we demonstrated that RasGRP1 is important for Erk1/2 in γδT cells following TCR stimulation. Thus, although additional RasGRPs and other guanine nucleotide-releasing factors, such as Sos, are expressed in γδT cells, they cannot fully compensate for the loss of RasGRP1 in Erk1/2 activation. Extending previous studies demonstrating that RasGRP1 is critical for positive selection of αβT cells (25, 27), we now find that RasGRP1 is also important for efficient β selection, as evidenced by the accumulation of DN3 cells and decreased DN4 thymocytes, suggesting that RasGRP1 participates in pre-TCR signaling.

In RasGRP1−/− mice, total and CD4−/CD8− DN and CD8SP γδT cell numbers in the thymus are comparable to those in WT controls. The virtually normal development of RasGRP1−/− γδT cells in total and CD4−/CD8− DN and CD8SP γδT cells is also consistent with the normal development of RasGRP1−/− γδT cells.

**FIGURE 5.** Requirement of RasGRP1 for TCR-induced Erk1/2 activation in γδT cells. (A) Altered RasGRP2 and RasGRP4 expression in RasGRP1 KO γδT cells. mRNA levels of RasGRP2 and RasGRP4 in sorted WT and RasGRP1 KO γδT cells were determined by real-time quantitative RT-PCR. (B) Decreased Erk1/2 activation in RasGRP1 KO γδT cells. Sorted γδT cells from WT and RasGRP1−/− mice were rested in PBS for 30 min and then left unstimulated or stimulated with an anti-CD3ε Ab for 5 min. Erk1/2 phosphorylation was determined by immunoblotting analysis. (C) Decreased Egr1/2 and Id3 expression in RasGRP1−/− γδT cells. RNA isolated from WT and RasGRP1−/− γδT cells were reverse transcribed, and mRNA levels of indicated molecules were determined by real-time quantitative RT-PCR. Data shown are representative of two experiments. (D) Slight increase in Vγ1Vδ6.3+ γδT cells in RasGRP1−/− mice. Upper panels, TCRγδ and TCRVδ6.3 staining of total thymocytes from WT and RasGRP1−/− mice. Lower panels, TCRVγ1.1 staining of gated TCRγδ+TCRVδ6.3+ cells. (E) Percentages of TCRVγ1.1+Vδ6.3+ cells within the γδT cells. Data shown are representative of two (A–C) or three (D, E) experiments.

**FIGURE 6.** Requirement for RasGRP1 in TCR-induced γδT cell proliferation. (A and B) CFSE dilution assay to assess γδT cell proliferation and death. CFSE-labeled purified WT and RasGRP1−/− γδT and αβT cells were left unstimulated or stimulated with a plate-bound anti-CD3ε Ab for 72 h. Cultured cells were stained with Live/Dead before being analyzed by flow cytometry. (A) Graphs show CFSE intensity on gated live γδT cells. (B) Bar graphs show death rate of cultured γδT cells. (C) Impaired Erk1/2 activation in both thymic and splenic γδT cells in the absence of RasGRP1. (D) Expression of costimulatory molecules and cytokine receptors by RasGRP1 KO γδT cells. Data shown are representative of two (A–C) or three (D) experiments.
T cells in mixed chimeric mice further supports the minimal role for RasGRP1 in gd T cell development. CD42CD82DN gd T cells, and CD8SP gd T cells in particular, expand in RasGRP12/2 peripheral lymphoid organs. However, such expansion of RasGRP12/2 gd T cells is not observed in mixed BM chimeric mice, suggesting that cell-extrinsic factors lead to the expansion of gd T cells in the peripheral lymphoid organs in RasGRP12/2 mice. These extrinsic factors could be cytokines produced by RasGRP12/2 conventional αβT cells under the lymphopenic environment or by other cell types. Furthermore, CD4Foxp3 regulatory T cell numbers in RasGRP12/2 mice were decreased (29), which could also contribute to peripheral gd T cell expansion.

It was reported that TCR signal strength influences αβT and gd T cell lineage commitment, with strong TCR signal and enhanced Erk1/2 activity favoring the gd T lineage (21–23). It is intriguing that gd T cell development is virtually intact in RasGRP12/2 mice, with an obvious decrease in Erk1/2 activation. One potential explanation is that the relative strengths of Erk1/2 signaling dictate gdT versus αβT differentiation. The commitment of the progenitor to the αβT lineage or cells that have committed to the αβT fate could be more sensitive to the decreased Erk1/2

FIGURE 7. Defective IL-17 production by RasGRP12/2 gd T cells. (A) WT and RasGRP12/2 thymocytes, splenocytes, and LN cells were stimulated with PMA and ionomycin in the presence of GolgiPlug for 5 h. Stimulated cells were cell surface stained for TCRβ and TCRγδ, followed by intracellular cytokine staining for IFN-γ and IL-17A. Dot plots show IL-17A and IFN-γ expression in gated total TCRβ+ TCRγδ+ cells. (B) IFN-γ and IL-17A expression in CD4+, CD8α+, and DN γδT cells. LN cells were similarly treated and stained as in (A), with the addition of CD4 and CD8α staining. Contour plots show IFN-γ and IL-17A staining in gated WT and RasGRP12/2 CD4+, CD8α+, and DN γδT cells. (C) γδT cell subsets based on ex vivo CD27 and CD44 staining. Contour plots show CD27 and CD44 expression in gated TCRβ+ TCRγδ+CD4+ CD8+ γδT cells and splenocytes from WT and RasGRP1 KO mice. (D) Differential effects of RasGRP1 deficiency on IL-17A production by CD27+ CD44+ γδT cells and IFNγ expression by CD27+CD44+ γδT cells. Data shown are representative of three experiments.
activity compared with those that are committing to or have committed to the γδT lineage. It is important to note that our data do not rule out a role for Erk1/2 in γδT cell development, because a low level of Erk1/2 phosphorylation can still be induced in RasGRPA+/− γδT cells. Furthermore, TCR stimulation induces stronger Erk1/2 activation in peripheral γδT cells than in thymic γδT cells, suggesting differential regulation of Erk1/2 activation between thymic and peripheral γδT cells. The low level of TCR-induced Erk1/2 activity in thymic γδT cells may suggest that developing γδT cells can be less sensitive to the decrease in Erk1/2 activation in the absence of RasGRPA1 than can peripheral γδT cells. Additionally, we recently demonstrated that, in addition to Erk1/2, enriched in γδT cells was reported to be able to expand CD8SP+CD44+ γδT cells. However, it is unclear how RasGRPA1 promotes IL-17 production by γδT cells. In αβT cells, mTOR complex 1 signaling is critical for Th17 differentiation (55, 56). Interestingly, RasGRPA1 is critical for mTOR activation in T cells by activating Ras-Erk1/2 signaling (42, 43). Future studies should determine whether RasGRPA1 promotes IL-17-producing γδT cells through inducing mTOR activation and whether RasGRPA1 can be a potential target for γδT cell-mediated diseases.

In summary, we identified several important functions of RasGRPA1 in T cells. We demonstrated that RasGRPA1 is involved in αβT cell maturation from the DN to DP stage, is important for TCR-induced Erk1/2 activation in both thymic and splenic γδT cells, is dispensable for overall γδT cell development but important for the generation/maintenance of IL-17-producing γδT cells, and, finally, is critical for TCR-induced γδT cell proliferation.

Acknowledgments
We thank Drs. Yuan Zhuang, Michael Kulis, and Tommy O’Brien for critically reviewing the manuscript and Nancy Martin and Mike Cook in the Duke Cancer Center Flow Cytometry Core Facility for sorting cells.

Disclosures
The authors have no financial conflicts of interest.

References

RasGRPA1 IN γδ T CELL GENERATION AND ACTIVATION


