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Critical Roles of RasGRP1 for Invariant NKT Cell Development

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The invariant NKT (iNKT) cell lineage contains CD4+ and CD4− subsets. The mechanisms that control such subset differentiation and iNKT cell maturation in general have not been fully understood. RasGRP1, a guanine nucleotide exchange factor for TCR-induced activation of the Ras–ERK1/2 pathway, is critical for conventional αβ T cell development but dispensable for generating regulatory T cells. Its role in iNKT cells has been unknown. In this study, we report severe decreases of iNKT cells in RasGRP1−/− mice through cell intrinsic mechanisms. In the remaining iNKT cells in RasGRP1−/− mice, there is a selective absence of the CD4+ subset. Furthermore, RasGRP1−/− iNKT cells are defective in TCR-induced proliferation in vitro. These observations establish that RasGRP1 is not only important for early iNKT cell development but also for the generation/maintenance of the CD4+ iNKT cells. Our data provide genetic evidence that the CD4+ and CD4− iNKT cells are distinct sublineages with differential signaling requirements for their development. The Journal of Immunology, 2011, 187: 000–000.

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Natural killer T cells are subsets of T cells coexpressing markers found on NK cells and T cells. Although rare in number, NK cells play important roles in immune responses and pathogenesis of disease (1–3). The invariant Vα14–Jα18 TCR (iVα14TCR)-expressing invariant NKT (iNKT) cells represent the major subset within the NKT cell lineage and are the best characterized (4, 5). The iVα14TCR recognizes both endogenous and synthetic glycolipids such as iGb3 and α-galactosylceramide (α-GalCer), respectively, presented by CD1d (6, 7). Use of CD1d tetramers loaded with α-GalCer has provided a pivotal tool to define iNKT cells and has allowed for the delineation of iNKT cell development into multiple developmental stages. The earliest iNKT cells (stage 0) are defined as CD24+CD44+NK1.1+, and such cells are extremely rare in the thymus. As iNKT cells mature, they downregulate CD24 expression and progress sequentially through stage 1 (CD24−CD44+ NK1.1+), stage 2 (CD24−CD44+ NK1.1−), and finally stage 3 (CD24−CD44+ NK1.1−) (8, 9). Further from these stage definitions, iNKT cells can also be divided into CD4+ and CD4− subsets that may branch out at stage 1 and represent two different sublineages of iNKT cells (10). However, genetic evidence supporting such sublineage definition remains quite rare.

The iVα14TCR is critical for iNKT cell development. Deficiency of the receptor or its ligand CD1d results in a failure to generate iNKT cells in mice (11–13). Upon TCR engagement, PLCγ1 plays a crucial role in TCR signaling by producing diacylglycerol (DAG) and inositol 1,4,5-triphosphate second messengers (14). Inositol 1,4,5-triphosphate activates the Ca2+-calcinurin–NFAT pathway, which has recently been demonstrated to be crucial for iNKT cell maturation via the transcription factor Egr2 (15). DAG activates the PKCθ–Carma1–Bcl10–Malt–IKK–NF-κB pathway. The NF-κB pathway is critical for iNKT cell ontogeny, as deficiencies of its different components have been shown to block iNKT cell development at various stages (16–21). DAG also associates with and activates RasGRP1, a guanine nucleotide releasing factor for Ras. RasGRP1 in turn activates the downstream Ras–ERK1/2–API pathway. The RasGRP1–Ras–ERK1/2 pathway is important for positive selection of conventional αβ T (cαβT) cells (22, 23). While uncontrolled DAG-mediated signaling due to absence of DAG kinases α and ξ causes severe defect of iNKT cell development (24), the role of RasGRP1 in iNKT cell development remains unclear. In this report, we demonstrate severe decreases of iNKT cells and a selective absence of the CD4+ subset of iNK-T cells in RasGRP1−/− mice. Our data not only reveal a critical role of RasGRP1 for early iNKT cell development but also provide genetic evidence that the CD4+ and CD4− subsets of iNKT cells are indeed distinct sublineages, as they have differential signaling requirements for their generation/maintenance.

Materials and Methods

Mice

The C57BL6/J and TCRβ−/−γ−/− mice were all purchased from The Jackson Laboratory. The RasGRP1−/− mice were previously described (22) and were back-crossed onto B6 background for nine generations. All mice were used according to a protocol approved by the Duke University Institutional Animal Care and Use Committee. Thymocytes and spleenocytes were prepared following standard procedures. Liver monocellular cells were isolated according to a published protocol (18).

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Abs and flow cytometry

Cells were stained with PE– or allophycocyanin–CD1d-Tet (National Institutes of Health Tetramer Core Facility) and fluorescence-conjugated anti-mouse CD24, CD45, NK1.1, CD4, CD8, TCRβ, CD45.1, CD45.2, Thy1.1, Thy1.2, CD1d, CD150 (SLAM), and Ly108 (SLAMF6) Abs (BioLegend) in PBS–2% FBS on ice for 30 min. Cell survival/death was determined by addition of the Live/Dead Fixable Violet Dead Cell Stain (Invitrogen) during the staining according to the manufacturer’s protocol. Dead cells stain positive for the Live/Dead Fixable Violet Dead Cell Stain. For Ki67 expression, cells were permeabilized using the Foxp3 staining kit (eBioscience) after cell surface staining, followed by staining with unconjugated anti-Ki67 (B56; BD Biosciences). An Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen) was used to detect anti-Ki67 Ab. Stained cells were collected on a FACSCanto II (BD Biosciences) and analyzed using the FlowJo software.

Enrichment of iNKT cells

Thymocytes (100 million) were resuspended in 500 μl IMDM with 10% FBS (IMDM-10) and were subjected to sequential addition of 5 μl Fc-blocker from the EasySep PE selection kit (Stem Cell Technologies) and 2.5 μl PE-CD1d-Tet. After incubation at room temperature for 15 min, cells were washed once with IMDM-10. The cells were resuspended in 500 μl IMDM-10 and mixed with 5 μl EasySep PE selection mixture. After incubation at room temperature for 15 min, 5 μl EasySep nanoparticles were added, and the mixture was incubated at room temperature for an additional 15 min. After addition of IMDM-10 to a total volume of 2.5 ml, cells in FACS tubes were inserted into the EasySep magnet and allowed to stand for 5 min. The unbound cells were discarded, and the bound cells were resuspended in 2.5 ml IMDM-10. After repeating magnetic enrichment for another time, the magnetic bound fractions were collected for staining and FACS analysis.

iNKT cell proliferation

Thymocytes from wild-type (WT) and RasGRP1−/− mice were labeled with 10 μM CFSE at room temperature for 9 min as previously described (25). Cells were seeded at 5 × 10^6 cells/ml in a 48-well plate and left unstimulated or stimulated with 125 ng/ml α-GalCer at 37°C for 72 h. Cells were then stained for TCRβ and allophycocyanin-conjugated CD1d-Tet before being analyzed by flow cytometry.

Bone marrow reconstitution

Recipient TCRβ−/−b−/− mice were sublethally irradiated (600 rad) 1 d before adoptive transfer. Ten million 1:1 mixed bone marrow (BM) cells from age- and sex-matched CD45.1−/− B6 and CD45.2−/− RasGRP1−/− mice were intravenously injected into the recipients. Alternatively, lethally irradiated (1100 rad) WT C57B6 mice were used as recipients and were reconstituted with Thy1.1−/−C57B6 (WT) and Thy1.2−/−RasGRP1−/− BM cells at 1:10 ratio. The resulting chimeric mice were analyzed 7 to 8 wk later.

Real-time PCR

Viable CD4^+CD8^+ double-positive (DP) thymocytes and TCRβ^−CD1d-Tet^+ iNKT cells from age- and sex-matched control or RasGRP1−/− mice

**FIGURE 1.** Critical role of RasGRP1 for iNKT cell development. A. Expression of RasGRP1 and RasGRP2 in WT iNKT cells. mRNA levels in FACS-sorted iNKT cells and coβT cells were determined by real-time PCR. B and C. Thymocytes, splenocytes, and liver mononuclear cells from RasGRP1−/− (KO) and RasGRP1−/− (Ctrl) littermates were subjected to flow cytometry analysis. Data shown are representative of five mice per group. B. Flow cytometry of cells stained with α-GalCer–loaded CD1d-tetramer (CD1d-Tet) and anti-TCRβ. C. Percentage (left) and number (right) of live CD1d-Tet^+TCRβ^+ cells (mean ± SEM). D. iNKT cells staining after enrichment for CD1d-Tet^+ cells with magnetic beads. Thymocytes from WT and RasGRP1−/− mice were stained with PE–CD1d-Tet. iNKT cells were enriched with a PE-enrichment kit. Top panels, TCRβ and CD1d-Tet staining of enriched cells. Bottom panels, CD24 expression on gated TCRβ^−CD1d-Tet^−NK1.1− cells. E. Total CD24^+CD45.1− iNKT cell numbers in WT and RasGRP1−/− thymi (n = 6). F. Assessment of iNKT cell development within CD24^+ iNKT cells. Dot plots show expression of CD44 and NK1.1 on CD1d-Tet^−CD24^+ gated live thymocytes. Bar figure shows percentages (mean ± SEM) of stages 1, 2, and 3 iNKT cells in RasGRP1−/− control mice. G. CD4 and CD8 staining of WT and RasGRP1−/− thymi. H. Ratios of iNKT cells to coβT cells in the thymus, spleen, and liver from WT and RasGRP1−/− mice. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test). Li, liver; Spl, spleen; Thy, thymus.
were sorted on a MoFlo Cell Sorter (Beckman Coulter), with postsort purity >98%, and lysed in TRIzol (Invitrogen). Total RNAs were extracted, and cDNAs were obtained using the Superscript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using the RealMasterMix (Eppendorf) and performed on the Mastercycler ep realplex² system (Eppendorf). Primers used for different genes are listed as follows: forward 5'-ACGCCTCTGGCATTTCTCG-3' and reverse 5'-ACATTGCCACACACCCCATTA-3'. BOR: forward 5'-GAACCAGAGACTGATTTG-3', reverse 5'-TTGGGAAAATCCCAACACATA-3'. RUNX1: forward 5'-CGAGGAGAAATGTTGACAC-3', reverse 5'-TTGGGAAAATCCCAACACATA-3'. cMyC: forward 5'-TGAGGCTGGATTTCCTTTG-3', reverse 5'-TTCCCTGCTGTTTGTCCTA-3'. PZA: forward 5'-GAACCCATATGTCTGCTG-3', reverse 5'-TGCTCATATTGGGAAG-3'. RasGRP1: forward 5'-AGCCCACCTTCTGTGACAAC-3', reverse 5'-ACATTGCACACAGCCCATTA-3'. RasGRP2: forward 5'-ACACTGCCACCTACATCTGT-3', reverse 5'-CAAAACGTACCTGGT-3'. RasGRP4: forward 5'-CTTCTTGCACTCGAACACCA-3', reverse 5'-GGTGGGACACTCGAACACCA-3'. RasGRP6: forward 5'-AGTCAGTTCGTGGAGGCCGGAAATC-3', reverse 5'-GCTCAAACTTTCAGAATCTACCGAC-3'. RasGRP7: forward 5'-AGGTTGTGCACTCGAACACCA-3', reverse 5'-GTGGCAGTTCACACCACAAG-3'. RasGRP8: forward 5'-GGTGGGACACTCGAACACCA-3', reverse 5'-GTGGCAGTTCACACCACAAG-3'. RasGRP9: forward 5'-CTTCTTGCACTCGAACACCA-3', reverse 5'-AGCCCACCTTCTGTGACAAC-3'. RasGRP10: forward 5'-ACATTGCACACAGCCCATTA-3', reverse 5'-CAAAACGTACCTGGT-3'. RasGRP11: forward 5'-CGAGGAGAAATGTTGACAC-3', reverse 5'-TTGGGAAAATCCCAACACATA-3'. RasGRP12: forward 5'-ACACTGCCACCTACATCTGT-3', reverse 5'-CAAAACGTACCTGGT-3'.

Assessment of Va–Ja recombination

Decreasing amounts of DNA template (100 ng, 33 ng, 11 ng) from sorted viable RasGRP1/− and RasGRP1+/− CD4+CD8+ thymocytes were used for semiquantitative PCR. The forward primer for Vox14 segment was 5' -ACACTGCCACCTACATCTGT-3'. The reverse primers for different Jα segments were as follows: Jα2 5'-GGTTTGCACTCGAACACCA-3'; Jα18 5'-GTAGAAAGAAACCTACTCACCA-3'; Jα56 5'-TGTCATCTCTCATGAAGCAC-3'. Primers for CD14 PCR (loading control) were as follows: forward 5'-GCTCAACTTTCAGAATCTACCGAC-3', reverse 5'-AGTCAAGTTCTGAGGCGGAAATC-3'.

Statistics

For statistical analysis, two-tailed Student t test was performed (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Critical role of RasGRP1 for iNKT cell development

The expression of RasGRP1 and the other RasGRP1 family members in iNKT cells has been unknown. We first examined their expression in iNKT cells. iNKT cells stained positive for both α-GalCer–loaded CD1d-Tet and TCRβ in thymocytes from WT mice. These iNKT cells were sorted by FACS, and mRNA levels of these genes were determined by real-time quantitative PCR. As shown in Fig. 1A, both RasGRP1 and RasGRP2 can be detected in coβT cells and iNKT cells. However, RasGRP3 and RasGRP4 were undetectable in iNKT cells (data not shown).

To determine the role of RasGRP1 for iNKT cell development, we analyzed mice deficient in RasGRP1. Thymocytes, splenocytes, and liver mononuclear cells were stained with CD1d-Tet, as well as other cell surface markers. As shown in Fig. 1B and 1C, CD1d-Tet+ TCRβ+ cells were decreased ~6- to 11-fold in RasGRP1−/− mice compared with that in RasGRP1+/− mice. To determine further

FIGURE 2. iNKT developmental defects in RasGRP1−/− mice are cell intrinsic. A–C. Generation and analysis of sublethally irradiated TCRβ−/−8−/− recipient mice reconstituted with WT (CD45.1+CD45.2+) and RasGRP1−/− (CD45.1−CD45.2+) BM at a 1:1 ratio. Chimeric mice were analyzed 7–8 wk after reconstitution. A. Expression of CD45.1 and CD45.2 on mixed WT and RasGRP1−/− BM cells before adoptive transfer. B. Analysis of coβT cells and non-T cells in recipient mice. Left panels show CD4 and CD8 staining of thymocytes and splenocytes from recipient mice. Middle and right panels show CD45.1 and CD45.2 staining in the DN, DP, and SP populations based on CD4 and CD8 expression. C. Analysis of iNKT cells in recipient mice. Left panels show CD45.1 and CD45.2 staining in the indicated organs from recipient mice. Middle and right panels show expression of CD1d-Tet and CD24 on gated CD45.1+CD45.2− WT and CD45.1−CD45.2+ RasGRP1−/− cells. D. Analysis of lethally irradiated WT C57B6 recipient mice reconstituted with WT (Thy1.1) and RasGRP1−/− (Thy1.2) BM cells at a 1:10 ratio. Left panel, Thy1.1 and Thy1.2 staining of recipient thymocytes. Middle and right panels, CD24 and CD1d-Tet staining as well as CD4 and CD8 staining gated on Thy1.1+ and Thy1.2+ thymocytes. E, CD1d expression on RasGRP1−/− and control CD4+CD8+ DP thymocytes. Data are representative of three (A–C) or two (D, E) experiments.

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whether RasGR1 is required for generation of stage 0 CD24+ iNKT cells, we enriched iNKT cells from WT and RasGR1−/− thymi using PE-CD1d-Tet and magnetic beads. As shown in Fig. 1D and 1E, the CD24+ iNKT cell number in RasGR1−/− thymi was 6-fold lower than that in WT control, indicating that RasGR1 is required for efficient generation of stage 0 iNKT cells. Further analysis of the CD1d-Tet+CD24+ iNKT cells revealed relative enrichment of CD44+ and CD45.1− populations but a decrease of the CD44+NK1.1+ population in RasGR1−/− mice (Fig. 1F). Together, these observations indicate that RasGR1 is critical for early iNKT cell development and is also involved in promoting iNKT maturation at later stages. Similar to a previous report (22), there was a severe protease in sorted CD4+CD8+ thymocytes from RasGR1−/− mice (Fig. 1F). Thus, the defect of iNKT cell development caused by RasGR1 deficiency was in parallel with that of coβT cells. However, the iNKT cells to coβT cell ratio was ∼7-fold lower in RasGR1−/− liver than in WT liver.

Cell intrinsic defect of developing RasGR1−/− iNKT cells

Because RasGR1 is expressed in multiple cell lineages and in iNKT cells are positively selected by engagement of i7α14TCR with CD1d expressed on DP thymocytes, we further investigated whether the developmental defects of RasGR1−/− iNKT cells are intrinsic. To this end, a 1:1 mixture of CD45.2+ RasGR1−/− and CD45.1+CD45.2+ WT BM cells were adoptively transferred to reconstitute TCRβ−/−δ−/− hosts. The recipients were analyzed 7 to 8 wk after reconstitution. Although a close to 1:1 ratio of WT and RasGR1−/− BM cells were injected into the recipients (Fig. 2A), only 15% of thymocytes from the recipients and <40% of total splenocytes and liver mononuclear cells were derived from RasGR1−/− origin (Fig. 2B, 2C). There were progressive decreases of representation by RasGR1−/− thymocytes as they mature from the CD4+CD8− double-negative (DN), the CD4+CD8+ DP, to the CD4+CD8− or CD4−CD8+ single-positive (SP) stage. The decrease was most severe within the RasGR1−/− CD4 SP and CD8 SP populations. Further analysis of non-T cells (DN) from splenocytes in the recipients revealed roughly equal contributions of WT and RasGR1−/− origins, suggesting that RasGR1 deficiency does not globally affect hematopoietic stem cell engraftment or early lymphoid progenitor cells and that RasGR1 may promote coβT cell development at multiple stages.

In the thymi of recipient mice, CD45.1+CD45.2+ WT CD1d-Tet+ iNKT cells could be easily detected. However, the CD45.2+ RasGR1−/− CD1d-Tet+ iNKT cells were virtually undetectable in the recipients (Fig. 2C). Similarly, few RasGR1−/− iNKT cells were observed in the spleen and liver as well. Because of underrepresentation of RasGR1−/− thymocytes in chimeric mice reconstituted with WT and RasGR1−/− BM cells at a 1:1 ratio, we further generated and analyzed chimeric mice reconstituted with BM cells from WT (Thy1.1) and RasGR1−/− (Thy1.2) mice at a 1:10 ratio. As shown in Fig. 2D, Thy1.1 and Thy1.2 staining of thymocytes from recipients displayed a close to expected ratio of cells originated from WT to RasGR1−/− BM cells. Severe decreases of Thy1.2+ RasGR1−/− iNKT cells as well as CD4

**FIGURE 3.** Increased death of iNKT cells in the absence of RasGR1. A. Semiquantitative PCR analysis of sorted CD4+CD8+ thymocytes from RasGR1−/− and RasGR1−/− mice with primers for Vα14-1x2, Vα14-1x18, Vα14-1x56, and CD14 (loading control). B. Expression of CD1d, SLAM (CD150), and SLAMF6 (Ly108) on CD4+CD8+ thymocytes from RasGR1−/− and RasGR1−/− mice. Data shown are representative of three mice per group. C. Percentages of cell death of CD1d-Tet+TCRβ−/− iNKT cells and CD1d-Tet− TCRβ−/− coβT cells from thymus (mean ± SEM, n = 4). D and E. Increased Ki67 expression in RasGR1−/− iNKT cells. Ki67 expression in iNKT cells gated from WT and RasGR1−/− thymocytes were determined by intracellular staining. D, Overlay of histogram for Ki67 expression of gated iNKT cells. E, Mean ± SEM of Ki67+ iNKT cells from WT and RasGR1−/− thymus (n = 5). F, Impaired proliferation of RasGR1−/− iNKT cells in response to α-GalCer stimulation in vitro. CFSE-labeled WT and RasGR1−/− thymocytes were left unstimulated or stimulated with 125 ng/ml α-GalCer at 37°C for 72 h. Cells were then stained for allophycocyanin–CD1d-Tet and TCRβ. Overlaid histograms show CFSE levels in gated WT and RasGR1−/− iNKT cells. G, Real-time PCR analysis of mRNA expression of various proteins in sorted CD4+CD8+ thymocytes from RasGR1−/− and RasGR1−/− mice. *p < 0.05, **p < 0.01 (Student t test).
and CD8 SP αβT cells were observed compared with their Thy1.1+ WT counterparts in the recipients. Together, these observations indicate that the developmental defects of RasGRP1<sup>−/−</sup> iNKT cells and αβT cells are cell intrinsic and cannot be rescued by WT thymocytes. CD1d expression on cortical thymocytes is critical for iNKT cell development (26, 27). No obvious difference was observed between RasGRP1<sup>+/−</sup> and control thymocytes (Fig. 2E). Together, these data indicate that RasGRP1 deficiency does not affect CD1d expression and rule out defective presentation by cortical thymocytes as a cause of defective iNKT cell development in RasGRP1<sup>−/−</sup> mice.

**Increased death of iNKT cells in the absence of RasGRP1**

Insufficient V<sub>α</sub>14-J<sub>α</sub>18 recombination has been shown to cause a severe developmental block early in iNKT development in some mouse models (28, 29). We detected similar levels of V<sub>α</sub>14 to J<sub>α</sub>18, J<sub>α</sub>2, or J<sub>α</sub>56 recombination in RasGRP1<sup>+/−</sup> and control thymocytes (Fig. 3A), ruling out the possibility that the deficiency of RasGRP1 somehow inhibited V<sub>α</sub>14-J<sub>α</sub>18 recombination. Beside V<sub>α</sub>14TCR, homotypic interactions of cell surface receptors Slamsf1 and Slamsf6 on thymocytes also play an essential role in iNKT development (30). No differences in the surface expression of these receptors were detected between RasGRP1<sup>+/−</sup> and RasGRP1<sup>−/−</sup> thymocytes (Fig. 3B). However, a significantly higher rate of cell death was observed in the RasGRP1<sup>−/−</sup> CD1d-Tet<sup>−</sup>TCR<sup>b</sup> iNKT cells as well as CD1d-Tet<sup>−</sup> TCR<sup>b</sup> αβT cells than in the RasGRP1<sup>+/−</sup> controls, suggesting that RasGRP1 is important for iNKT and αβT cell survival, and increased death of these cells may contribute to the developmental defects in RasGRP1<sup>−/−</sup> mice (Fig. 3C). Ki67 expression is usually correlated with cell division. Freshly isolated RasGRP1<sup>−/−</sup> iNKT cells displayed a higher level of Ki67 staining compared with control iNKT cells (Fig. 3D, 3E), suggesting increased homeostatic proliferation of RasGRP1<sup>−/−</sup> iNKT cells.

**FIGURE 4.** Selective absence of CD4<sup>+</sup> iNKT cells in RasGRP1<sup>−/−</sup> mice. A, Expression of CD4 on CD1d-Tet<sup>+</sup> CD24<sup>−</sup>-gated cells from thymus, spleen, and liver. B, Percentage of CD4<sup>+</sup> in the CD1d-Tet<sup>+</sup>CD24<sup>−</sup> iNKT cells in the indicated organs (n = 4). C, Expression of CD4 and NK1.1 on CD1d-Tet<sup>+</sup>CD24<sup>−</sup>-gated thymocytes with various cell surface phenotypes. D, Percentage of CD4<sup>+</sup> iNKT cells in different stages (n = 4). E, Schematic illustration of RasGRP1 for αβT and iNKT cell development. RasGRP1 plays a critical role for positive selection of both αβT and iNKT cells. RasGRP1 is crucial for the generation/maintenance of CD4<sup>+</sup> iNKT cells. In addition, RasGRP1 may promote late-stage iNKT cell maturation. ***p < 0.001 (Student t test).
of these molecules were detected between cMyc (36), and HEB (28), are all critical for early iNKT cell development (37). No obvious differences in mRNA expression of these molecules were detected between RasGRP1+/- and RasGRP1−/− DP thymocytes (Fig. 3G), ruling out that RasGRP1 deficiency may affect iNKT cell development through modulating mRNA expression of these molecules. However, expression of PLZF, a transcription factor critical for the development of CD4+ iNKT cells (38, 39), was much lower in RasGRP1+/- iNKT cells than in RasGRP1−/− control, which might contribute to the relative enrichment of CD24+,CD44+ iNKT cells in RasGRP1−/− mice.

Selective absence of CD4+ iNKT cells in RasGRP1−/− mice

It has been demonstrated that whereas stage 0 iNKT cells all express CD4 (8), the presence of CD4+ iNKT cells can be observed in thymus at later stages or in the periphery. Although accumulating evidence has revealed that the CD4+ and CD4− iNKT cells are functionally distinct (10, 40, 41), the developmental relationship between these two subsets is not well understood. Recently published data show that the CD4− NK1.1− cells appeared to be precursors of the CD4+ NK1.1+ iNKT cells in the thymus. A revised model of thymic iNKT development was proposed in which the CD4+ and CD4− subsets represent two distinct sublineages of iNKT cells, whose divergence appears to occur at stage 1 when the CD4− iNKT cells are first observed (10). However, genetic evidence supporting such lineage definition is rare, and mechanisms directing such lineage differentiation are not well defined. Strikingly, a dramatic decrease in the percentage of CD4+ subset was observed in the RasGRP1−/− iNKT cells in thymus, spleen, and liver (Fig. 4A, 4B). When assessing the CD4 expression pattern at each iNKT developmental stage in RasGRP1−/− thymus, there is a progressive increase of the CD4− subset as the iNKT cells mature. About 10, 25, and 40% of stage 1, stage 2, and stage 3 iNKT cells are CD4−. However, in RasGRP1−/− iNKT cells, ~90% of stage 2 and stage 3 iNKT cells were CD4−, yet no difference in CD4+/CD4− ratio was observed at stage 1 compared with RasGRP1+/- (Fig. 4C, 4D). Thus, besides promoting early iNKT cell development, RasGRP1 is selectively required for the maturation and/or maintenance of the CD4+CD44+ iNKT cells.

Discussion

RasGRP1 promotes positive selection of coβT cells, particularly those expressing TCR with low affinity to self peptide–MHC complex (22). Positive selection of thymocytes with relative high affinity to self peptide–MHC complex, including regulatory T cells and some innate CD8+ T cells, is less dependent on RasGRP1 (42, 43). We have demonstrated in this study that RasGRP1 plays crucial roles in iNKT cell development and is important for the generation and/or maintenance of CD4+ iNKT cells (Fig. 4E). At present, it is still unclear how RasGRP1 promotes coβT and iNKT cell maturation. The increased death of RasGRP1-deficient coβT cells and iNKT cells suggests that RasGRP1 may promote normal development of iNKT and coβT cells by enhancing their survival. Of note, in addition to activating the Ras–ERK1/2 pathway in thymocytes after TCR engagement (22), we have recently found that RasGRP1 is also critical for TCR-induced activation of PI3K/Akt and the mammalian target of rapamycin (mTOR) (44). Both PI3K/Akt and mTOR are important regulators for cell survival, growth, and metabolism (45–47). It is likely that RasGRP1 may promote iNKT cells and coβT cell maturation through multiple mechanisms.

The CD4+CD44+ iNKT cells are selectively or more severely affected than the CD4+CD44+ iNKT cells by RasGRP1 deficiency, suggesting that these two subsets of cells may signal differently. In RasGRP1-deficient thymocytes, TCR-induced activation of Ras/ERK1/2, PI3K/Akt, and mTOR is greatly decreased but not completely abolished (44). The exact differences of these signaling events between the CD4+ iNKT T cells and CD4− iNKT T cells, as well as the effect of RasGRP1 deficiency on the activation of these signaling pathways in iNKT cells, are hard to assess because these cells are rare. At present, it is unclear whether the CD4+CD44+ iNKT cells are independent of or less dependent on one or multiple signaling pathways downstream of RasGRP1 or whether they use other guanine nucleotide exchange factors such as Sos to activate these downstream signaling molecules. However, RasGRP1 promotes Sos to induce Ras activation (48). TCR-induced Ras/ERK1/2 activation in RasGRP1−/− iNKT cells is likely decreased, and the CD4+CD44+ and the CD4−CD44+ iNKT cells probably have differential requirement for the Ras–ERK1/2 pathway. In addition to RasGRP1, deficiency of the transcription factor GATA-3 also causes a severe decrease of CD4+ iNKT cells in mice (49). Together, these observations provide genetic evidence that the CD4+ and CD4− iNKT cells are distinct sublineages with differential signaling/transcription factor requirements for their development. Further studies are required to determine whether RasGRP1 and GATA-3 may regulate each other to promote CD4+ iNKT cell development.

It is important to note that our data appear to contradict a previous report that the Ras–Mek1/2–ERK1/2 pathway is dispensable for NKT cell development (50). In that study, dominant-negative Ras and Mek1, specifically expressed in thymocytes, cause severe decreases of CD4+CD8− and CD4+CD8+ SP thymocytes. However, NK1.1+TCR+ T cells were reported to be normal. Because CD1d-GalCer tetramer was not available at that time, the effects of dnRas/dnMek1 on iNKT cell development remain unclear. However, we did observe sharp decrease of NK1.1+TCR+ cells in RasGRP1−/− mice as well (data not shown). The discrepancy between these two studies could result from less complete abolishment of the Ras–ERK1/2 signaling in thymocytes of the dnRas/dnMek transgenic mice than in the RasGRP1−/− mice, some unknown effects of dnRas/dnMek1 transgenes on the cells, or variegate expression pattern due to the integration site effects on the transgenes. Additionally, RasGRP1 deficiency and dnRas or dnMek1 may differentially affect signaling pathways such as the PI3K/Akt, mTOR, and other yet to be identified signaling pathways that may play different roles in iNKT cell development.

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Disclosures

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