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# Critical roles of RasGRP1 for invariant natural killer T cell development

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## Abstract

The invariant NKT (*i*NKT) cell lineage contains $CD4^+$ and $CD4^-$ subsets. The mechanisms that control such subset differentiation and *i*NKT cell maturation in general have not been fully understood. RasGRP1, a guanine nucleotide exchange factor for T cell receptor-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 *i*NKT cells has been unknown. Here we report severe decreases of *i*NKT cells in *RasGRP1*$^{-/-}$ mice through cell intrinsic mechanisms. In the remaining *i*NKT cells in *RasGRP1*$^{-/-}$ mice, there is a selective absence of the $CD4^+$ subset. Furthermore, *RasGRP1*$^{-/-}$ *i*NKT cells are defective in T cell receptor induced proliferation *in vitro*. These observations establish that RasGRP1 is not only important for early *i*NKT cell development, but also for the generation/maintenance of the $CD4^+$ *i*NKT cells. Our data provides genetic evidence that the $CD4^+$ and $CD4^-$ *i*NKT cells are distinct sub-lineages with differential signaling requirements for their development.

## INTRODUCTION

Natural killer T (NKT) cells are subsets of T cells co-expressing markers found on NK cells and T cells. While rare in number, NKT cells play important roles in immune responses and pathogenesis of disease (1-3). The invariant Vα14-Jα18 T cell receptor (*i*Vα14TCR) expressing NKT (*i*NKT) cells represent the major subset within the NKT cell lineage and are the best characterized (4, 5). The *i*Vα14TCR recognizes both endogenous and synthetic glycolipids such as iGB3 and α-galactosyl ceramide (α-GalCer), respectively, presented by CD1d (6, 7). Use of CD1d tetramers loaded with α-GalCer has provided a pivotal tool to define *i*NKT cells and has allowed for the delineation of *i*NKT cell development into multiple developmental stages. The earliest *i*NKT cells (stage 0) are defined as $CD24^+CD44^-NK1.1^-$, and such cells are extremely rare in the thymus. As *i*NKT cells mature, they down-regulate CD24 expression and progress sequentially through stage 1

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(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, *i*NKT cells can also be divided into CD4$^+$ and CD4$^-$ subsets that may branch out at the stage 1 and represent two different sub-lineages of *i*NKT cells (10). However, genetic evidence supporting such sub-lineage definition remains quite rare.

The *i*Vα14TCR is critical for *i*NKT cell development. Deficiency of the receptor or its ligand CD1d results in a failure to generate *i*NKT 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-tris-phosphate ($IP_3$) second messengers (14). $IP_3$ activates the $Ca^{++}$-calcineurin-NFAT pathway, which has been recently demonstrated to be crucial for *i*NKT cell maturation via the transcription factor Egr2 (15). DAG activates the PKCθ-Carma1/Bcl10/Malt-IKK-NFκB pathway. The NFκB pathway is critical for *i*NKT cell ontogeny, as deficiencies of its different components have been shown to block *i*NKT 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-AP1 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 diacylglycerol kinases α and ζ causes severe defect of iNKT cell development (24), the role of RasGRP1 in *i*NKT cell development remains unclear. In this report, we demonstrate severe decreases of *i*NKT cells and a selective absence of the CD4$^+$ subset of *i*NKT cells in RasGRP1$^{-/-}$ mice. Our data not only reveals a critical role of RasGRP1 for early *i*NKT cell development, but also provide genetic evidence that the CD4$^+$ and CD4$^-$ subsets of *i*NKT cells are indeed distinct sublineages since they have differential signaling requirements for their generation/maintenance.

## MATERIALS AND METHODS

### Mice

The C57BL6/J and *TCRβ*$^{-/-}$*δ*$^{-/-}$ mice were all purchased from the Jackson Laboratory. The *RasGRP1*$^{-/-}$ mice were previously described (22) and were backcrossed onto B6 background for 9 generations. All mice were used according to a protocol approved by the Duke University Institute Animal Care and Use Committee. Thymocytes and splenocytes were prepared following standard procedures. Liver mononuclear cells were isolated according to a published protocol (18).

### Antibodies and flow cytometry

Cells were stained with PE- or APC-CD1d-Tet (NIH tetramer core facility) and fluorescence-conjugated anti-mouse CD24, CD44, NK1.1, CD4, CD8, TCRβ, CD45.1, CD45.2, Thy1.1, Thy1.2, CD1d, CD150 (SLAM), and Ly108 (SLAMF6) antibodies (BioLegend) in PBS-2% FBS on ice for 30 minutes. Cell survival/death was determined by addition of the Live/Dead® Fixable Violet Dead Cell Stain (L/D, Invitrogen) during the staining according to the manufacturer's protocol. Dead cells stain positive for L/D. For Ki67 expression, cells were permeabolized using the Foxp3 staining kit (eBioscience) after cell surface staining, followed by staining with unconjugated anti-Ki-67 (B56, BD Biosciences). An Alexa Fluor® 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen) was used to detect anti-Ki67 antibody. Stained cells were collected on FACSCanto™ II (BD Biosciences) and analyzed using the Flowjo software.

### Enrichment of *i*NKT cells

Thymocytes were resuspended in 500 μl IMDM with 10% FBS (IMDM-10) and were sequentially added with 5μl Fc-blocker from the EasySep PE selection kit (Stem Cell

Technologies) and 2.5 μl of PE-CD1d-Tetramer. After incubation at room temperature (RT) for 15 minutes, cells were washed once with IMDM-10. The cells were resuspended in 500 μl of IMDM-10 and mixed with 5 μl of EasySep PE selection cocktail. After incubation at RT for 15 minutes, 5 μl of EasySep nanoparticles were added and the mixture was incubated at RT for additional 15 minutes. After addition of IMDM-10 to a total volume of 2.5 ml, cells in FACS tubes were inserted into the EasySep magnet and let stand for 5 minute. 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 bounding fractions were collected for staining and FACS analysis.

### *i*NKT cell proliferation

Thymocytes from WT and *RasGRP1*$^{-/-}$ mice were labeled with 10 μM CFSE at RT for 9 minutes as previously described (25). Cells were seed at 5 ×10$^6$ cells/ml in a 48-well plate plate and left unstimulated or stimulated with 125 ng/ml α-GalCer at 37°C for 72 hours. Cells were then stained for TCRβ and APC-conjugated CD1d-Tet before analyzed by flow cytometry.

### Bone marrow reconstitution

Recipient *TCRβ*$^{-/-}$*δ*$^{-/-}$ mice were sublethally irradiated (600 rad) one day 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 weeks later.

### Real time PCR

Viable CD4$^+$CD8$^+$ double positive (DP) thymocytes and TCRβ$^+$CD1dTet$^+$ *i*NKT cells from age- and sex-matched control or *RasGRP1*$^{-/-}$ mice were sorted on MoFlo Cell Sorter (Beckman Coulter), with post-sort purity>98%, and lysed in Trizol (Invitrogen). Total RNAs were extracted, and cDNAs were obtained using the Superscript III First-Strand Synthesis System (Invitrogen). Realtime PCR was prepared using the RealMasterMix (Eppendorf) and performed on the Mastercycler® ep realplex$^2$ system (Eppendorf). Primers used for different genes are listed as following. SAP: forward 5'-acgcctctgcagtatccagt-3', reverse 5'-ttcttcatggtgcattcagg-3'; Fyn: forward 5'-caagccaagcagtgtttgaa-3', reverse 5'-acattgcacacagcccatta-3'; RORγt: forward 5'-cgactggaggaccttctacg-3', reverse 5'-ttggcaaactccaccacata-3'; RUNX1: forward 5'-gcaggacgaatcacactgaa-3', reverse 5'-tggcatctctcatgaagcac-3'; cMyc: forward 5'-tgaaggctggatttcctttg-3', reverse 5'-ttctcttcctcgtcgcagat-3'; HEB: forward 5'-aggtatggatgagcgtggag-3', reverse 5'-agccttcgtgggttcctaat-3'; PLZF: forward 5'-tgcgcagctatatttgcagt-3', reverse 5'-tgtggctcttgagtgtgctc-3'; RasGRP1: forward 5'-agcccaccttctgtgacaac -3', revers3 5'-cttcttgcactcgaacacca-3'; RasGRP2: forward 5'-gggcttcgtacacaacttcc-3', reverse 5'-gtggcagttcacaccacaag-3'.

### Assessment of Vα-Jα recombination

Decreasing amounts of DNA template (100ng, 33ng, 11ng) from sorted viable *RasGRP1*$^{+/-}$ and *RasGRP1*$^{-/-}$ CD4$^+$CD8$^+$ thymocytes were used for semi-quantitative PCR. The forward primer for Vα14 segment was 5'-acactgccacctacatctgt-3'. The reverse primers for different Jα segments were: Jα2 5'-ggttgcaaatggtgccactt-3'; Jα18 5'-gtagaaagaaacctactcacca-3'; Jα56

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5’-tgtcatcaaaacgtacctggt-3’. Primers for CD14 PCR (loading control) were: forward 5’-gctcaaactttcagaatctaccgac-3’, reverse agtcagttcgtggaggccggaaatc-3’.

### Statistics

For statistic analysis, two-tail Student t-test was performed. *, $p<0.05$. **, $p<0.01$, ***, $p<0.001$.

## RESULTS

### Critical role of RasGRP1 for *i*NKT cell development

The expression of RasGRP1 and the other RasGRP1 family members in iNKT cells has been unknown. We first examined their expression in *i*NKT cells. *i*NKT cells stained positive for both α-Galcer loaded CD1d tetramer (CD1d-Tet) and TCRβ in thymocytes from wild-type (WT) mice. These *i*NKT 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 cαβT cells and *i*NKT cells. However, RasGRP3 and RasGRP4 were undetectable in *i*NKT cells (Data not shown).

To determine the role of RasGRP1for *i*NKT 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 Figure 1B-1C, $CD1dTet^+TCR\beta^+$ cells were decreased about 6 – 11 fold in *RasGRP1*$^{-/-}$ mice as compared to *RasGRP1*$^{+/-}$ mice. To further determine whether RasGRP1 is required for generation of stage 0 $CD24^+$ *i*NKT cells, we enriched *i*NKT cells from WT and *RasGRP1*$^{-/-}$ thymus using PE-CD1d-Tet and magnetic beads. As shown in Figure 1D-1E, the $CD24^+$ *i*NKT cell number in *RasGRP1*$^{-/-}$ thymi was 6-fold lower than WT control, indicating that RasGRP1 is required for efficient generation of stage 0 *i*NKT cells. Further analysis of the $CD1dTet^+CD24^-$ *i*NKT cells revealed relative enrichment of $CD44^-NK1.1^-$ and $CD44^+NK1.1^-$ populations, but a decrease of the $CD44^+NK1.1^+$ population in *RasGRP1*$^{-/-}$ mice (Fig. 1F). Together, these observations indicate that RasGRP1 is critical for early *i*NKT cell development and is also involved in promoting *i*NKT maturation at later stages. Similar to a previous report (22), there was a severe decrease of cαβT cells and about 50% decrease of total thymocyte number in *RasGRP1*$^{-/-}$ mice (Fig. 1G and data not shown). The ratios of *i*NKT cells to cαβT cells in *RasGRP1*$^{-/-}$ thymus and spleen were similar to those of WT controls (Fig. 1H). Thus, the defect of *i*NKT cell development caused by RasGRP1 deficiency was in parallel with that of cαβT cells. However, the *i*NKT cells to cαβT cell ratio was about seven-fold lower in *RasGRP1*$^{-/-}$ liver than in WT liver.

### Cell intrinsic defect of developing *RasGRP1*$^{-/-}$ *i*NKT cells

Since RasGRP1 is expressed in multiple cell lineages and *i*NKT cells are positively selected by engagement of *i*Vα14TCR with CD1d expressed on DP thymocytes, we further investigated whether the developmental defects of *RasGRP1*$^{-/-}$ *i*NKT cells are intrinsic. To this end, a 1:1 mixture of $CD45.2^+$ *RasGRP1*$^{-/-}$ and $CD45.1^+CD45.2^+$ WT BM cells were adoptively transferred to reconstitute *TCRβ*$^{-/-}$*δ*$^{-/-}$ hosts. The recipients were analyzed seven to eight weeks after reconstitution. Although a close to 1:1 ratio of WT and *RasGRP1*$^{-/-}$ BM cells were injected into the recipients (Fig. 2A), only 15% of thymocytes from the recipients and less than 40% of total splenocytes and liver mononuclear cells were derived from *RasGRP1*$^{-/-}$ origin (Fig. 2B-2C). There were progressive decreases of representation by *RasGRP1*$^{-/-}$ 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 *RasGRP1*$^{-/-}$ CD4 SP and CD8 SP populations. Further analysis of non-T cells (DN) from splenocytes in the recipients revealed a roughly equal

contributions of WT and *RasGRP1*$^{-/-}$ origins, suggesting that RasGRP1 deficiency does not globally affect hematopoietic stem cell engraftment or early lymphoid progenitor cells and that RasGRP1 may promote cαβT cell development at multiple stages.

In the thymi of recipient mice, CD45.1$^{+}$CD45.2$^{+}$ WT CD1dTet$^{+}$ *i*NKT cells could be easily detected. However, the CD45.2$^{+}$ *RasGRP1*$^{-/-}$ CD1dTet$^{+}$ *i*NKT cells were virtually undetectable in the recipients (Fig. 2C). Similarly, few *RasGRP1*$^{-/-}$ *i*NKT cells were observed in the spleen and liver as well. Due to under representation of *RasGRP1*$^{-/-}$ thymocytes in chimeric mice reconstituted with WT and *RasGRP1*$^{-/-}$ BM cells at 1:1 ratio, we further generated and analyzed chimeric mice reconstituted with BM cells from WT (Thy1.1) and *RasGRP1*$^{-/-}$ (Thy1.2) mice at 1:10 ratio. As shown in figure 2D, Thy1.1 and Thy1.2 staining of thymocytes from recipients displayed a close to expected ratio of cells originated from WT to *RasGRP1*$^{-/-}$ BM cells. Severe decreases of Thy1.2$^{+}$ *RasGRP1*$^{-/-}$ *i*NKT cells as well as CD4 and CD8 SP cαβT cells were observed as compared with their Thy1.1$^{+}$ WT counterparts in the recipients. Together, these observations indicate that the developmental defects of *RasGRP1*$^{-/-}$ *i*NKT cells and cαβT cells are cell-intrinsic and cannot be rescued by WT thymocytes. CD1d expression on cortical thymocytes are critical for *i*NKT cell development (26, 27). No obvious difference was observed between *RasGRP1*$^{-/-}$ 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 *i*NKT cell development in *RasGRP1*$^{-/-}$ mice.

## Increased death of *i*NKT cells in the absence of RasGRP1

Insufficient Vα14-Jα18 recombination has been shown to cause a severe developmental block early in *i*NKT development in some mouse models (28, 29). We detected similar levels of Vα14 to Jα18, Jα2, or Jα56 recombination in *RasGRP1*$^{+/-}$ and *RasGRP1*$^{-/-}$ CD4$^{+}$CD8$^{+}$ DP thymocytes (Fig. 3A), ruling out the possibility that the deficiency of RasGRP1 somehow inhibited Vα14-Jα18 recombination. Beside *i*Vα14TCR, homotypic interactions of cell surface receptors Slamsf1 and Slamsf6 on thymocytes also play an essential role in *i*NKT development (30). No differences in the surface expression of these receptors were detected between *RasGRP1*$^{+/-}$ and *RasGRP1*$^{-/-}$ thymocytes (Fig. 3B). However, a significantly higher rate of cell death was observed in the *RasGRP1*$^{-/-}$ CD1d-Tet$^{+}$TCRβ$^{+}$ *i*NKT cells as well as CD1d-Tet$^{-}$TCRβ$^{+}$ cαβT cells than in the *RasGRP1*$^{+/-}$ controls, suggesting that RasGRP1 is important for *i*NKT and cαβT cell survival, and increased death of these cells may contribute to the developmental defects in *RasGRP1*$^{-/-}$ mice (Fig. 3C). Ki67 expression is usually correlated with cell division. Freshly isolated *RasGRP1*$^{-/-}$ *i*NKT cells displayed a higher level of Ki67 staining compared with control *i*NKT cells (Fig. 3D-3E), suggesting increased homeostatic proliferation of *RasGRP1*$^{-/-}$ *i*NKT cells *in vivo*, likely due to the T cell lymphopenic environment in these mice. We further used CFSE-dilution assay to examine whether RasGRP1 regulates TCR induced *i*NKT cell activation. As shown in Figure 3F, WT but not *RasGRP1*$^{-/-}$ *i*NKT cells proliferated following α-GalCer stimulation for 72 hours *in vitro* (Fig. 3F). Together, these data suggest that RasGRP1 is important for *i*Vα14TCR-induced *i*NKT cell activation.

Signaling proteins SAP (31, 32) and Fyn (33, 34), as well as several transcription factors, such as RORγt (29, 35), Runx1 (35), cMyc (36), and HEB (28), are all critical for early *i*NKT 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 *i*NKT cell development through modulating mRNA expression of these molecules. However, expression of PLZF, a transcription factor critical for the development of CD44$^{+}$ *i*NKT cells (38, 39), was much lower in *RasGRP1*$^{-/-}$ *i*NKT cells than in *RasGRP1*$^{+/-}$ control, which might contribute to the relative enrichment of CD24$^{-}$CD44$^{-}$ *i*NKT cells in *RasGRP1*$^{-/-}$ mice.

### Selective absence of CD4$^+$ *i*NKT cells in *RasGRP1*$^{-/-}$ mice

It has been demonstrated that, while stage 0 *i*NKT cells all express CD4 (8), the presence of CD4$^-$ *i*NKT cells can be observed in thymus at later stages or in the periphery. While accumulating evidence has revealed that the CD4$^+$ and CD4$^-$ *i*NKT 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$^+$ *i*NKT cells in the thymus. A revised model of thymic *i*NKT development was proposed in which the CD4$^-$ and CD4$^+$ subsets represent two distinct sub-lineages of *i*NKT cells, whose divergence appears to occur at stage 1 when the CD4$^-$ *i*NKT 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*$^{-/-}$ *i*NKT cells in thymus, spleen, and liver (Fig. 4A-4B). When assessing the CD4 expression pattern at each *i*NKT developmental stage in *RasGRP1*$^{+/-}$ thymus, there is a progressive increase of the CD4$^-$ subset as the *i*NKT cells mature. About 10%, 25% and 40% of stage 1, stage 2 and stage 3 *i*NKT cells are CD4$^-$. However, in *RasGRP1*$^{-/-}$ *i*NKT cells, about 90% of stage 2 and stage 3 *i*NKT cells were CD4$^-$, yet no difference in CD4$^+$/CD4$^-$ ratio was observed at stage 1 as compared to *RasGRP1*$^{+/-}$ (Fig. 4C-4D). Thus, besides promoting early *i*NKT cell development, RasGRP1 is selectively required for the maturation and/or maintenance of the CD4$^+$CD44$^+$ *i*NKT cells.

## Discussion

RasGRP1 promotes positive selection of cαβT 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 here that RasGRP1 plays crucial roles in *i*NKT cell development and is important for the generation and/or maintenance of CD4$^+$ *i*NKT cells (Fig. 4E). At present, it is still unclear how RasGRP1 promotes αβT and *i*NKT cell maturation. The increased death of RasGRP1 deficient cαβT cells and *i*NKT cells suggests that RasGRP1 may promote normal development of *i*NKT and cαβT cells by enhancing their survival. Of note, in addition to activating the Ras-Erk1/2 pathway in thymocytes following 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 *i*NKT cells and cαβT cell maturation through multiple mechanisms.

The CD4$^+$CD44$^+$ *i*NKT cells are selectively or more severely affected than the CD4$^-$CD44$^+$ *i*NKT 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$^+$*i*NKT T cells and CD4$^-$*i*NKT T cells, as well as the effect of RasGRP1 deficiency on the activation of these signaling pathways in *i*NKT cells, are hard to assess since these cells are rare. At present, it is unclear whether the CD4$^-$CD44$^+$ *i*NKT cells are independent or less dependent of one or multiple signaling pathways downstream of RasGRP1 or they utilize 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*$^{-/-}$ *i*NKT cells is likely decreased and the CD4$^+$CD44$^+$ and the CD4$^+$CD44$^-$ *i*NKT cells probably have differential requirement for the Ras/Erk1/2 pathway. In addition to RasGRP1, deficiency of the transcription factor GATA-3 also cause a severe decrease of CD4$^+$ *i*NKT cells in mice (49). Together, these observations provide genetic evidence that

the $CD4^+$ and $CD4^-$ *i*NKT cells are distinct sublineages with differential signaling/transcription factor requirements for their development. Further studies are required to determine whether RasGRP1 and GATA3 may regulate each other to promote $CD4^+$ *i*NKT 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 $CD4^-CD8^+$ single positive thymocytes. However, $NK1.1^+TCR\beta^+$ T cells were reported to be normal. Since CD1d-Galcer tetramer was not available at that time, the effects of dnRas/dnMek1 on *i*NKT cell development remain unclear. However, we did observe sharp decrease of $NK1.1^+TCR\beta^+$ 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 for *i*NKT cell development.

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## Abbreviations

| | |
|---|---|
| **α-Galcer** | α-galactosyl ceramide |
| **DAG** | Diacylglycerol |
| ***i*NKT** | invariant natural killer T cell |
| ***i*Vα14TCR** | invariant Vα14-Jα18 T cell receptor |
| **cαβT** | conventional αβ T cells |
| **DP** | double positive |
| **SP** | single positive |
| **Li** | Liver |
| **Spl** | spleen |
| **Thy** | thymus |

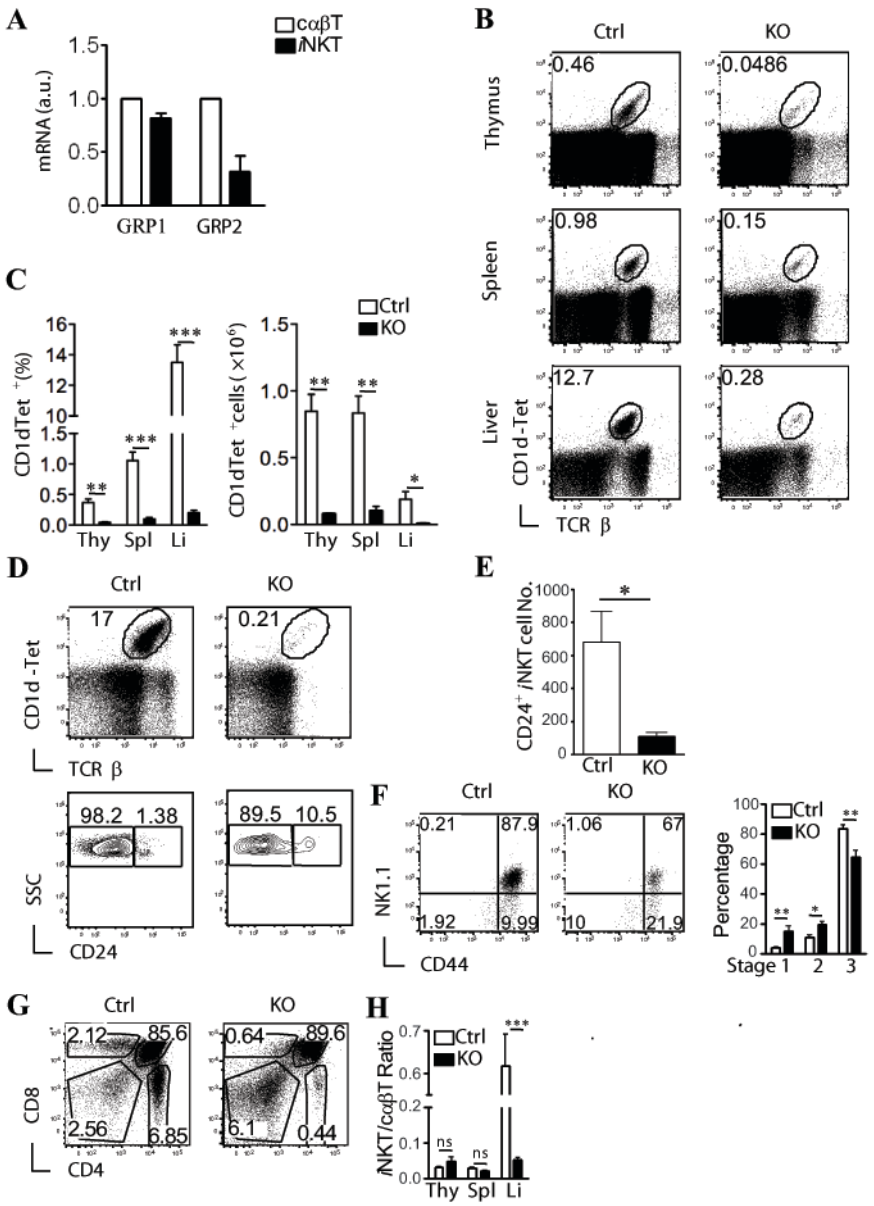


**Figure 1. Critical role of RasGRP1 for *i*NKT cell development**

(A) Expression of RasGRP1 and RasGRP2 in WT *i*NKT cells. mRNA levels in FACS sorted *i*NKT cells and cαβT cells were determined by real-time PCR. (B-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) *i*NKT cells staining after enrichment for CD1d-Tet$^{+}$ cells with magnetic beads. Thymocytes from WT and RasGRP1$^{-/-}$ mice were stained with PE-CD1d-Tet. *i*NKT 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$^{+}$CD44$^{-}$NK1.1$^{-}$ *i*NKT cell numbers in WT and *RasGRP1*$^{-/-}$ thymi (n=6). (F) Assessment of *i*NKT cell development within CD24$^{-}$ *i*NKT 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 *i*NKT cells in RasGRP1$^{-/-}$ and control mice. (G) CD4 and CD8 staining of WT and *RasGRP1*$^{-/-}$ thymi. (H) Ratios of *i*NKT cells to cαβT cells in the thymus (Thy), spleen (Spl), and liver (Li) from WT and *RasGRP1*$^{-/-}$ mice. *p<0.05; **, p<0.01; ***, p<0.001 (Student t-test).

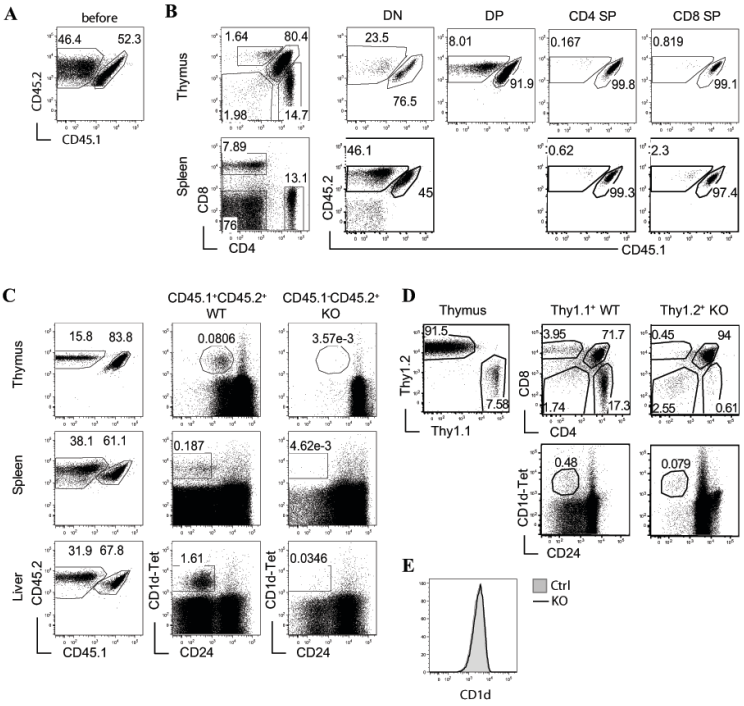


**Figure 2. *i*NKT developmental defects in *RasGRP1*$^{-/-}$ mice are cell-intrinsic**

(A-C) Generation and analysis of sublethally irradiated TCRβ$^{-/-}$δ$^{-/-}$ recipient mice reconstituted WT (CD45.1$^{+}$CD45.2$^{+}$) and *RasGRP1*$^{-/-}$ (CD45.1$^{-}$CD45.2$^{+}$) bone marrow (BM) at 1:1 ratio. Chimeric mice were analyzed 7-8 weeks after reconstitution. (A) Expression of CD45.1 and CD45.2 on mixed WT and *RasGRP1*$^{-/-}$ BM cells before adoptive transfer. (B) Analysis of cαβT 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 *i*NKT 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 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.

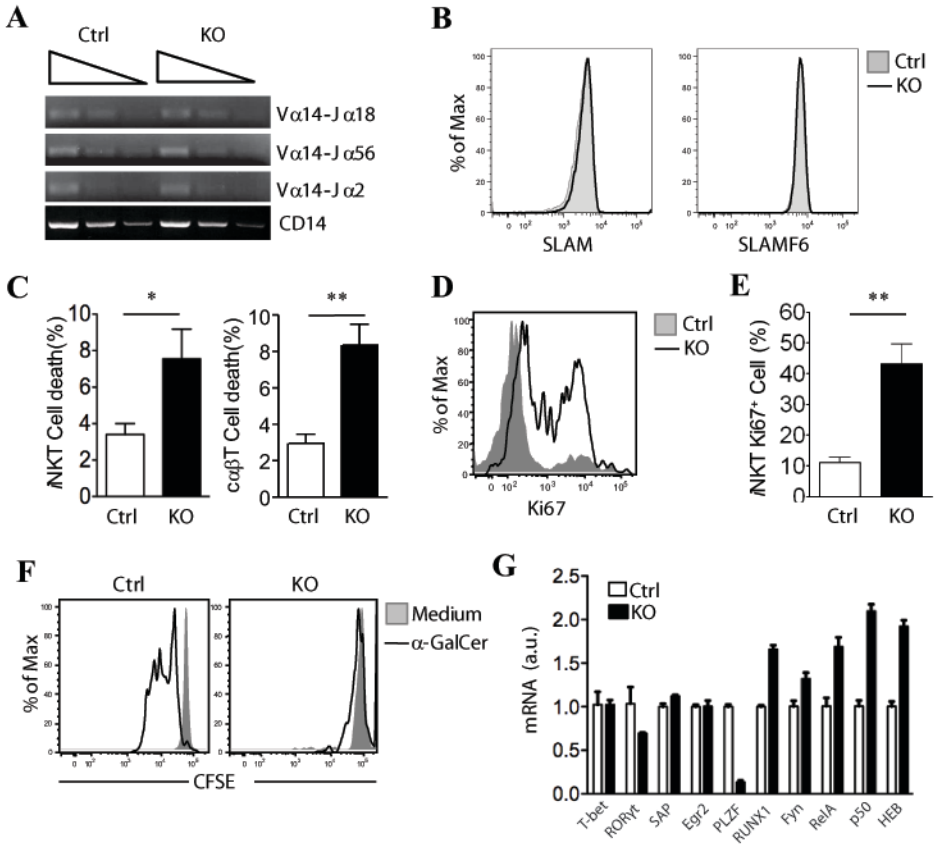


**Figure 3. Increased death of *i*NKT cells in the absence of RasGRP1**

(A) Semi-quantitative PCR analysis of sorted $CD4^+CD8^+$ thymocytes from *RasGRP1*$^{+/-}$ and *RasGRP1*$^{-/-}$ mice with primers for Vα14-Jα2, Vα14-Jα18, Vα14-Jα56, and CD14 (loading control). (B) Expression of CD1d, SLAM (CD150), and SLAMF6 (Ly108) on $CD4^+CD8^+$ thymocytes from *RasGRP1*$^{+/-}$ and *RasGRP1*$^{-/-}$ mice. Data shown are representative of three mice per group. (C) Percentages of cell death of CD1d-$Tet^+TCR\beta^+$ *i*NKT cells and CD1d-$Tet^-TCR\beta^+$ cαβT cells from thymus (mean ± SEM, n=4). (D, E) Increased Ki67 expression in RasGRP1$^{-/-}$ *i*NKT cells. Ki67 expression in *i*NKT cells gated from WT and RasGRP1$^{-/-}$ thymocytes were determined by intracellular staining. (D) Overlay of histogram for Ki67 expression of gated *i*NKT cells; (E) Mean ± SEM of $Ki67^+$ *i*NKT cells from WT and *RasGRP1*$^{-/-}$ thymus (n=5). (F) Impaired proliferation of *RasGRP1*$^{-/-}$ *i*NKT cells in response to α-Galcer stimulation *in vitro*. CFSE-labeled WT and *RasGRP1*$^{-/-}$ thymocytes were left unstimulated or stimulated with 125 ng/ml α-Galcer at 37°C for 72 hours. Cells were then stained for APC-CD1d-Tet and TCRβ. Overlaid histograms show CFSE levels in gated WT and *RasGRP1*$^{-/-}$ *i*NKT cells. (G) Real-time PCR analysis of mRNA expression of various proteins in sorted $CD4^+CD8^+$ thymocytes from *RasGRP1*$^{+/-}$ and *RasGRP1*$^{-/-}$ mice. *p<0.05; **, p<0.01 (Student t-test).

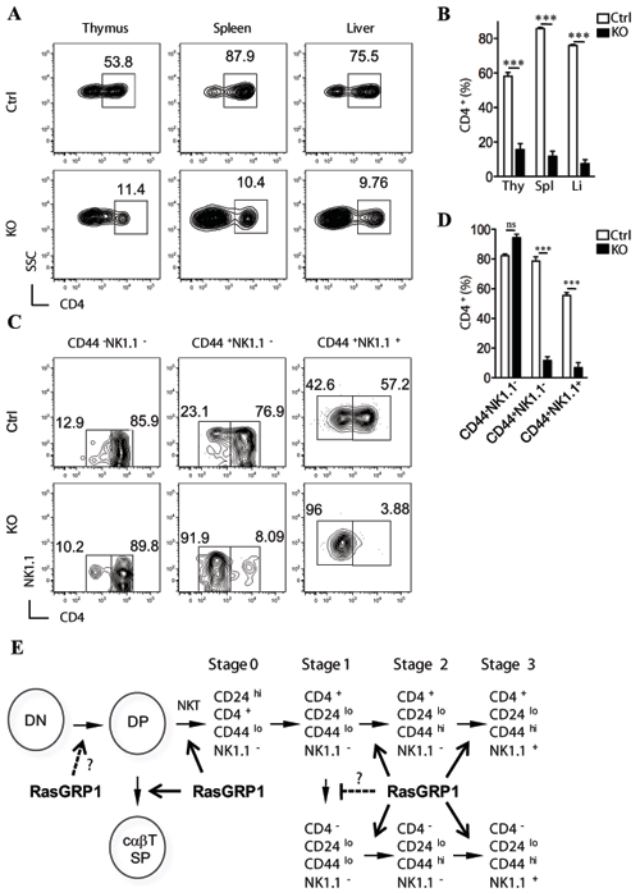


**Figure 4. Selective absence of CD4$^+$ *i*NKT cells in *RasGRP1*$^{-/-}$ mice**

(A) Expression of CD4 on CD1d-Tet$^+$CD24$^-$ gated cells from thymus, spleen, and liver. (B) Percentage of CD4$^+$ in the CD1d-Tet$^+$CD24$^-$ *i*NKT cells the indicated organs (n=4). (C) Expression of CD4 and NK1.1 on CD1d-Tet$^+$CD24$^-$ gated thymocytes with various cell surface phenotypes. (D) Percentage of CD4$^+$ *i*NKT cells in different stages (n=4). (E) Schematic illustration of RasGRP1 for cαβT and *i*NKT cell development. RasGRP1 plays a critical role for positive selection of both cαβT and *i*NKT cells. RasGRP1 is crucial for the generation/maintenance of CD4$^+$ *i*NKT cells. In addition, RasGRP1 may promote late stage *i*NKT cell maturation. ***, p<0.001 (Student t-test).