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The Ras-guanyl nucleotide exchange factor RasGRP1 is an important link between TCR-mediated signaling and the activation of Ras and its downstream effectors. RasGRP1 is especially critical for the survival and differentiation of developing thymocytes whereas negative selection of thymocytes bearing an autoreactive TCR appears to be RasGRP1 independent. Despite apparently normal central tolerance, RasGRP1−/− mice spontaneously acquire an acutely activated and proliferating CD4 T cell population that exhibits characteristics of T cell exhaustion, including strong expression of programmed cell death-1. To elucidate the basis for RasGRP1−/− CD4 T cell immune activation, we initiated a series of adoptive transfer experiments. Remarkably, the copious amounts of cytokines and self-Ags present in hosts made lymphopenic through irradiation failed to induce the majority of RasGRP1−/− CD4 T cells to enter cell cycle. However, their infusion into either congenitally T cell- or T/B cell-deficient recipients resulted in robust proliferation and L-selectin down-regulation. These findings imply that the activation and proliferation of RasGRP1−/− CD4 T cells may be dependent on their residence in a chronically immunocompromised environment. Accordingly, bacterial and viral challenge experiments revealed that RasGRP1−/− mice possess a weakened immune system, exhibiting a T cell-autonomous defect in generating pathogen-specific T cells and delayed pathogen clearance. Collectively, our study suggests that chronic T cell immunodeficiency in RasGRP1−/− mice may be responsible for CD4 T cell activation, proliferation, and exhaustion. The Journal of Immunology, 2007, 179: 2143–2152.
are driving fast T cell proliferation in congenitally T cell-deficient animals.

The loss of T cell immunity is a common occurrence during chronic viral infections in both mice and humans (8). Recent evidence suggests that continual exposure to cognate Ag results in the overstimulation of virus-specific T cells and the development of an “exhausted” memory T cell phenotype. In contrast to acute infection, memory T cells derived from chronic infection exhibit Ag dependency, limited self-renewal capacity, diminished cytokine production, and reduced cytotoxicity (9). Therefore, these functional impairments displayed by chronically activated T cells may contribute to the failure to clear virus. Programmed death-1 (PD-1), a negative regulator of activated T cells (10), is strongly up-regulated on exhausted virus-specific CD8 T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice and HIV infection in humans (11, 12). Because blockade of PD-1 interaction with its ligand PD-L1 can restore function in exhausted CD8 T cells (11), it makes the case that PD-1 is not simply indicative of an exhausted state but also plays a key role in its maintenance.

In thymocytes, Sos (13) and RasGRP1 (14), two Ras-guanine-nucleotide exchange factors, link Ras and MAPK activation to TCR signal transduction with their respective functions dependent on relocating to membranes by two distinct mechanisms (15). RasGRP1 mobilizes to membranes by binding the phospholipase Cγ1 product diacylglycerol through its C1 domain whereas Sos is recruited to the phosphorylated adaptor molecule linker for activated T cells by way of its association with the Src homology 2-domain-containing protein Grb2. RasGRP1−/− thymocytes show signs of reduced TCR signaling (14, 16, 17) and a selective impairment of positive but not negative selection (16). Therefore, mice lacking RasGRP1 serve as a model lacking a positive regulator of TCR signaling. RasGRP1−/− mice exhibit a marked T cell lymphopenia (14, 16), likely a consequence of decreased single-positive (SP) thymocyte maturation and T cell hyporesponsiveness (14, 16, 17). Paradoxically, a recently described novel mouse strain called RasGRP116/18 (lymphoproliferation-autoimmunity glomerulonephritis), bearing a spontaneous mutation in RasGRP1, develops an autoimmune syndrome resembling systemic lupus erythematosus (SLE), exhibiting massive lymphoproliferation, high levels of serum autoantibodies and, eventually, advanced disease that required euthanasia (18). Therefore, RasGRP1 signaling may be critical for both thymocyte maturation and T cell tolerance.

In this study, we report that although RasGRP1−/− mice remain T cell lymphopenic and free of overt disease until at least 1 year of age, they possess a population of proliferating CD4 T cells that display an exhausted phenotype, characteristic of chronic infection (8). Adoptive transfer experiments suggest that chronic immunodeficiency and foreign Ags might be responsible for inducing RasGRP1−/− CD4 T cell proliferation rather than autoreactivity to self-Ags. Supporting the notion of a dysfunctional immune system, bacterial and viral challenge experiments revealed that RasGRP1−/− mice exhibited impaired T cell responses and a delay in pathogen clearance. Lastly, we find that chronic T cell immunodeficiency in RasGRP1−/− mice is likely a consequence of RasGRP1 protein loss in thymocytes and/or T cells rather than due to defects in innate immunity. In conclusion, these investigations highlight the roles of RasGRP1 in determining a normal immune status and as an essential regulator of adaptive T cell immunity toward experimental infection.

Abbreviations used in this paper: PD-1, programmed cell death 1; PD-L1, PD-L1 ligand 1; DP, double-positive thymocyte; SP, single-positive thymocyte; LCMV, lymphocytic choriomeningitis virus; LN, lymph node; MLN, mesenteric LN; SLE, systemic lupus erythematosus; LLO, listeriolysin O.

### Materials and Methods

#### Mice

C57BL/6J (B6), B6.PL-Thy1.1/Cy (Thy 1.1*), B6.SJL-PpcepPep3/Bojyl (Ly 5.1*), B6.RAG-1−/−, and B6.TCRO−/− mice were acquired from The Jackson Laboratory. RasGRP1−/− breeder mice were provided by J. C. Stone (University of Alberta, Alberta, Canada) and bred onto a B6 background at least seven generations. To generate Thy1.1 2C TCR-transgenic animals, the 2C TCR transgene was bred onto the B6.PL-Thy1.1/Cy background (H-2b, Thy1.1*). All studies followed guidelines set by the Animal Care Committee at the University of British Columbia in conjunction with the Canadian Council on Animal Care.

#### Flow cytometry

Abs against CD4 (GK1.5), CD8 (53-6.7), CD25 (MEL-14), CD25 (PC61.5), CD69 (H1.2F3), CD45.1 (A20), Thy1.1 (HS51), CD44 (IM7), CD62L (MEL-14), CD127 (AKR34), PD-1 (J43), PD-L1 (MIHS), PD-L2 (TY25), TNF-α (MP6-XT22), and IFN-γ (XMG1.2) were purchased from eBioscience. Annexin V-FITC, anti-Fas (DX2), anti-Fasl (MFL3), anti-Ki-67 Abs (B56), and anti-TCR β screening panel (no. 0143KK) reagent sets were purchased from BD Biosciences.

#### Adoptive transfer experiments

Wild-type splenic and lymph node T cells were purified from Thy1.1* animals and labeled with 1 μM CFSE (Molecular Probes) as previously described (20). Approximately 2 × 10^6 purified wild-type (polyclonal) Thy1.1* T cells or 1 × 10^6 Thy1.1* 2C TCR CD8 T cells were i.v. injected into Thy1.2* recipients, either irradiated (600 rad) wild-type B6 or nonirradiated wild-type B6, B6.RasGRP1−/−, B6.RAG-1−/−, and B6.TCRO−/− mice. Conversely, ~2 × 10^6 CFSE-labeled RasGRP1−/− T cells (Thy1.2*) were transferred into either B6, Thy1.1+, irradiated B6.Thy1.1*, or B6.RAG-1− hosts. Splenocytes were recovered 1 wk post-transfer and proliferation of donor cells assessed by flow cytometry using a FACSCalibur (BD Biosciences).

#### Bacterial and viral infections

Mice were infected i.v. with ~10,000 CFU of a recombinant strain of *Listeria monocytogenes* engineered to express the 2C TCR agonist peptide SIYVYYGL (J. Prietel, L. Zwenicz, H. Shen, and H. Teh, manuscript in preparation). For viral infection, mice were injected i.p. with ~100,000 PFU of LCMV-Armstrong. Splenic viral titers were determined as described previously (21). For wild-type T cell infusion into RasGRP1−/− mice, animals, purified (1 × 10^7 million) Ly5.1* T cells (~55-45% ratio of CD4 vs CD8 T cells) were i.v. injected into RasGRP1−/− deficient animals 1 day before infection with either rLM-SIY or LCMV.

#### Detection of IFN-γ production by intracellular flow cytometry

Splenocytes were harvested from mice at either day 7 (rLM-SIY) or day 8 (LCMV) postinfection, processed through metal mesh to generate single-cell suspensions, and subjected to RBC lysis by ammonium chloride treatment. Splenocytes were cultured for 5 h in 96-well, flat-bottom plates, at a concentration of 2–4 × 10^6 cells/well, in 0.2 ml of complete medium supplemented with 1 μl/ml Golgi Plug (contains brefeldin A; BD Biosciences) to block cytokine secretion. Cells were stimulated with a concentration of 1 μM for the MHC class I peptides (SIY, SIYRYYGL; GP34–43, KAVYNFATC; GF34–43, AVYNFATCG; NP93–104, FQPQNGQFE; GP76–86, SP76–86, SGVVNPQYGC; NP95–106, YTVYKVPLN; OVA257–264, SIINFEKL) and 10 μM for the I-Ab MHC class II peptides (LLO190–201, NEKYAQAY; GLK226–234, PFIYAL; SVV257–264, YILNQPSL). Splenocytes were incubated for 5 h in a 24-well plate that had been precoated with 10 μg/ml anti-CD3ε (145-2C11) Ab. After culture, cells were fixed for 15 min in 2% paraformaldehyde/PBS solution, permeabilized for 15 min with 0.2% Tween 20PBS and stained with anti-CD4-allophycocyanin, anti-CD8-PE-Cy5, and anti-IFN-γ-APC/Fluorescein isothiocyanate (FITC) Abs (eBioscience). Data were acquired using CellQuest software (BD Biosciences) and analyzed with FCSExpress (www.fcsexpress.com). All peptides were synthesized at the University of British Columbia’s Nuclelic Acid Research Service Unit.
focus on RasGRP1-lele to the C57BL/6J (B6) genetic background. In this report, we report the phenotype of RasGRP1-deficient mice (14). To account for the limited positive selection in RasGRP1-deficient animals, it had been proposed that thymocytes capable of being selected without RasGRP1 must express strongly self-reactive TCRs to overcome their signaling deficits (16–18). Notably, autoreactive CD4 T cells were suspected to be the root cause of massive lymphoproliferation and an underlying autoimmune disorder in RasGRP1-deficient mice (18). In a recent report, we focus on RasGRP1-deficient mice that have been bred at least seven generations onto the B6 mouse background.

B6-backcrossed RasGRP1-deficient mice appear healthy until at least 1 year of age and do not develop massive lymphoproliferation. Sampling of 2- to 4-mo-old RasGRP1-deficient mice revealed that they remain T cell lymphopenic; the recovery of both CD4 and CD8 T cells from spleens and pooled lymph nodes (LN) was reduced vs age-matched wild-type mice (Fig. 1A). Comparison of secondary lymphoid organs revealed that RasGRP1-deficient mice have similar-sized spleens with respect to wild type (Fig. 1B). Curiously, RasGRP1-deficient mice possess small peripheral LN such as axillary, brachial and inguinal

**Results**

RasGRP1-deficient CD4 T cells exhibit markers of acute activation, exhaustion, proliferation, and spontaneous apoptosis

The impaired T cell development results in a T cell lymphopenia, exhibiting a 10-fold decreased abundance of peripheral T cells, in 1-mo-old RasGRP1-deficient mice (14). To account for the limited positive selection in RasGRP1-deficient animals, it had been proposed that thymocytes capable of being selected without RasGRP1 must express strongly self-reactive TCRs to overcome their signaling deficits (16–18). Notably, autoreactive CD4 T cells were suspected to be the root cause of massive lymphoproliferation and an underlying autoimmune disorder in RasGRP1-deficient mice (18). In this report, we focus on RasGRP1-deficient mice that have been bred at least seven generations onto the B6 mouse background.

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**FIGURE 1.** Peripheral CD4 T cells from RasGRP1-deficient mice display signs of activation, exhaustion, proliferation, and spontaneous apoptosis. A, RasGRP1-deficient animals possess a reduced frequency and numbers of peripheral CD4 and CD8 T cells. Decreased splenic CD4 and CD8 αβ TCR+ T cell numbers in mice lacking RasGRP1. The single (+) and double asterisk (**) represent p values, using an unpaired, two-tailed Student’s t test, calculated at p < 0.02 and p < 0.005, respectively. B, Comparison of spleens, PLNs (brachial LN is shown), and MLN between wild-type and age-matched RasGRP1-deficient mice. C, RasGRP1-deficient CD4 and CD8 αβ TCR+ T cells (bold line) possess markers of memory and acute activation. Thin line, shaded histograms indicate staining pattern of wild-type T cell counterparts. D, RasGRP1-deficient CD4 T cells (bold line) express elevated levels of PD-1 and its ligand PD-L1 as compared with wild type (thin line). Shaded histograms represent background autofluorescence of unstained cells. E, An increased frequency of RasGRP1-deficient CD4 T cells exhibit expression of the proliferation-associated nuclear Ag Ki-67. F, RasGRP1-deficient CD4 T cells bind high levels of apoptotic marker annexin V.

**Direct ex vivo CTL assays**

After 7 days postinfection with rLM-SIY, splenic CD8 T cell effectors were isolated by staining total splenocytes with rat anti-mouse CD4 (GK1.5) Abs and subsequently depleted of CD4+ and surface Ig+ cells with anti-mouse (and rat-reactive) Ig-linked Dynabeads (catalog no. 110.02; Dynal Biotech). The target EL-4 cell line was labeled with 51Cr, pulsed with SIY peptide, washed, and incubated with various numbers of effectors as previously described (20).

**FIGURE 2.** Aberrant-positive selection in RasGRP1-deficient mice results in a small population of SP thymocytes that bear a naive cell surface phenotype. A, Regions used for gating are indicated in the developmental profiles from wild-type and mutant mice. B, Expression of the CD5, CD44, CD62L, and CD69 differentiation markers are shown for wild-type (thin line) and RasGRP1-deficient (bold line) DP, CD4 SP, and CD8 SP thymocyte subpopulations. Shaded histograms represent autofluorescence of unstained thymocyte subpopulations.
that CD8 T cells (Thy 1.1) observations). In addition, a large proportion of RasGRP1 played diminished capacities to undergo homeostatic expansion and
raning from chronic viral infections (8). Therefore, we sought to de-
mits altered as

FIGURE 3. The cellular environment within RasGRP1-deficient animals promotes T cell expansion. A, Purified wild-type T cells (Thy 1.1+) were labeled with CFSE and adoptively transferred into normal C57BL/6J (B6), irradiated B6, normal B6.RasGRP1−/− and B6.TCRβ−/− mice. Splenocytes were harvested 1-wk posttransfer and the proliferation of donor CD4 and CD8 T cells measured by flow cytometry. B, Same type of experiment as in A except that contour plots are presented, displaying CD62L expression as a function of CFSE fluorescence. C, Same experiment as in A except that 2C CD8 T cells (Thy 1.1+) were adoptively transferred into the indicated host animals and donor T cell proliferation tracked by gating on Thy1.1+CD8+ 2C TCR+ cells.

LNs whereas mesenteric LNs (MLN) from the mutant mice were enlarged as compared with age-matched wild-type animals (Fig. 1B). Next, flow cytometric analyses revealed that RasGRP1−/− CD4 and CD8 T cells express very high levels of CD44 as compared with wild type regardless of whether they were isolated from the spleen, peripheral LN, or MLN (Fig. 1C and our unpublished observations). In addition, a large proportion of RasGRP1−/− CD4 T cells also display signs of acute activation (CD69high, CD127low, CD62Llow, Fashigh, Faslhigh). The activated and memory phenotype for RasGRP1−/− T cells is particularly conspicuous because our TCR-transgenic studies demonstrated that central tolerance was not affected by RasGRP1 deficiency and that RasGRP1−/− T cells displayed diminished capacities to undergo homeostatic expansion and respond to cognate Ag (16).

The activated state of RasGRP1−/− CD4 T cells is reminiscent of the exhausted phenotype described in mice and humans suffering from chronic viral infections (8). Therefore, we sought to determine whether RasGRP1−/− T cells expressed PD-1, a molecule that is strongly expressed by exhausted but not normal T cells (10). Strikingly, a large fraction of RasGRP1−/− CD4 T cells possess high levels of PD-1 on their surface (Fig. 1D). In addition, PD-L1, a PD-1 ligand that is constitutively expressed by most splenocytes (10), is markedly up-regulated by RasGRP1−/− CD4 T cells whereas it is more modestly elevated by mutant CD8 T cells (Fig. 1D). By contrast, expression of PD-L2 does not appear to be altered in RasGRP1−/− mice (our unpublished observations). Next, we examined whether T cell activation in RasGRP1−/− mice was linked with cellular proliferation (Fig. 1E). Indeed, RasGRP1−/− CD4 T cells exhibit an elevated frequency of cells bearing the

proliferation-associated nuclear Ag Ki-67 as compared with wild type (13.3 vs 5.0%). Because elevations in CD44 levels are intimately linked with peripheral T cell expansion (2), Ki-67 staining associates with increased CD44 expression in both wild-type and mutant mice (Fig. 1E). The difference in Ki-67 expression between wild-type and mutant CD4 T cells is significantly less pronounced when comparisons are based on the CD44high subsets (wild type = 13.4%; mutant = 15.7%). Notably, the CD44 expression profiles shown for wild-type and mutant T cells (Fig. 1E) is altered as compared with Fig. 1C because it was performed after formaldehyde fixation and permeabilization with methanol to facilitate the detection of the intracellular Ag Ki-67 (see Materials and Methods). Studies have shown that formaldehyde/methanol treatment of cells results in a reduction in the intensity of CD44 staining, as detected by the clone IM7 mAb, and an increase in background cell staining levels (22). Subsequently, we sought to determine whether RasGRP1−/− CD4 T cells show signs of accelerated cell death because elevated Fas/FasL levels and exhausted T cell phenotypes are associated with increased rates of T cell apoptosis (Fig. 1F). Staining with the apoptotic marker annexin V revealed that RasGRP1−/− CD4 T cells are strongly reactive toward this reagent (RasGRP1−/− = 76.0% vs RasGRP1−/− = 24.1%). Collectively, these findings suggest that RasGRP1−/− CD4 T cells are highly activated, cycling, and apoptotic as compared with wild type.

RasGRP1−/− CD4 SP thymocytes display a naive cell surface phenotype

Mice deficient in RasGRP1 exhibit severely diminished numbers of mature SP thymocytes demonstrating that this molecule plays a
critical role in thymopoiesis (14, 16, 18) (Fig. 2A). To examine whether RasGRP1−/− CD4 and CD8 T cells spontaneously acquire a memory phenotype from their development in the thymus, we stained thymocytes with Abs specific for CD4, CD8, and TCRβ to identify mature (TCRβ+) SP (CD4+CD8− or CD4−CD8+) thymocyte subpopulations. In stark contrast to peripheral RasGRP1−/− T cells, RasGRP1−/− SP thymocytes express abnormally low amounts of CD44 and CD69 as compared with their wild-type counterparts (Fig. 2B). However, in concordance with SP thymocyte maturation, RasGRP1−/− CD4 SP thymocytes bear equivalent expression of CD5, a marker of TCR signaling during positive selection (23), as well as similar levels of CD62L and Bcl-2 as compared with wild-type CD4 SP thymocytes (Fig. 2B and our unpublished observations). Strikingly, the CD44high phenotype of most RasGRP1−/− CD4 SP thymocytes contrasts with the elevated CD44 expression levels previously reported for RasGRP1muCD4 SP thymocytes (18). To explain the contradiction between these findings, we hypothesize that the massive lymphoproliferation and lymphocytic tissue infiltration observed in RasGRP1mu animals (18) results in activated peripheral CD4 T cells also infiltrating the thymus. Because B6-backcrossed RasGRP1−/− mice remain lymphopenic, fewer RasGRP1−/− CD4 T cells likely escape to the thymus and contaminate the CD4 SP electronic gate. Therefore, we argue that RasGRP1 deficiency supports the development of naive CD4 SP thymocytes and that their conversion to a CD44high phenotype in the periphery may result from homeostatic pressures.

**RasGRP1−/− host animals support rapid donor T cell proliferation**

A hypothesis for the origin of memory phenotype (CD44high) RasGRP1-deficient T cells is that they are the product of slow homeostatic proliferation that result in the conversion of naive T cells into memory T cells. Because a substantially reduced number of SP thymocytes develop and are exported to the peripheral lymphoid organs in RasGRP1−/− mice (16), the few mature SP thymocytes that immigrate to the periphery are subjected to a T cell lymphopenic environment. Therefore, the availability of IL-7 and self-MHC interactions in RasGRP1−/− mice may be well-suited for inducing the peripheral T cell expansion. To test this hypothesis, we adoptively transferred equivalent numbers of wild-type T cells (Thy1.2+), labeled with the mitotic tracker CFSE, into RasGRP1−/−, wild-type B6, irradiated B6 (600 rad) and TCRα−/− host animals for a 1-wk period (Fig. 3A). As expected, the majority of CD4 and CD8 T cells recovered from normal (lymphoreplete) mice had undergone slow expansion whereas those from irradiated recipients had undergone slow expansion that is characteristic of homeostatic proliferation, still retaining some fluorescence imparted by CFSE. In contrast, the outcome is substantially different when T cells are transferred into congenitally T cell-deficient TCRα−/− or RAG-1−/− mice (6, 7). In these chronically immunodeficient animals, which are completely devoid of any αβ TCR+ cells, some donor T cells underwent “typical” homeostatic proliferation, cycling one to four times per week, while other donor T cells divide rapidly, greater than eight times within a week, and completely lost their CFSE fluorescence. The massive growth of these rapidly dividing cells results in their increased numbers and representation when looking at the distribution of donor cell CFSE fluorescence 1 wk posttransplantation (TCRα null; Fig. 3A). Interestingly, the donor cell division history in RasGRP1−/− recipients revealed a unique CFSE profile bearing similarities to both TCRα−/− and irradiated B6 hosts: a rapidly dividing population (52% of CD4+ and 37% of CD8+ T cells) and a slowly dividing population (21% of CD4+ and 50% of CD8+ T cells), respectively. These findings demonstrate that the lymphopenia present within RasGRP1−/− mice promotes spontaneous peripheral T cell expansion.
Foreign Ags, derived from commensal microbes, are thought to be responsible for the rapid naive T cell expansion observed after transfer of naive T cells into chronically immunodeficient mice RAG-1⁻/⁻ and TCRα⁻/⁻ mice (7). To support the idea that these rapidly dividing T cells in RasGRP1⁻/⁻ recipients are undergoing an Ag-driven differentiation program, we charted CD62L (L-selectin), a marker that is down-modulated upon acute activation, vs CFSE fluorescence after 1-wk residence in vivo (Fig. 3B). Strikingly, a large proportion of the CFSElow CD4 (69%) and CD8 (47%) T cells recovered from RasGRP1⁻/⁻ mice have low CD62L expression. By comparison, T cells residing in irradiated B6 recipients maintain high levels of CD62L while the majority of T cells recovered from RAG-1⁻/⁻ hosts have down-regulated this marker (Fig. 3B). Next, we examined whether RasGRP1⁻/⁻ mice could also promote expansion of TCR-transgenic T cells by infusing wild-type 2C TCR-transgenic CD8 T cells (Thy1.1⁺), labeled with CFSE, into normal B6, irradiated B6, RAG-1⁻/⁻, and RasGRP1⁻/⁻ host animals and measuring cellular proliferation 1 wk later (Fig. 3C). Notably, RasGRP1⁻/⁻ recipients induce slow 2C T cell proliferation but this amount is considerably less than both RAG-1⁻/⁻ and irradiated B6 mice (97 and 87%, respectively). Moreover, the observation that 2C T cells do not undergo rapid proliferation in either RasGRP1⁻/⁻ or RAG-1⁻/⁻ hosts suggests that this type of T cell expansion may apply to only a subset of T cells with unique TCR specificity. Together, these results suggest that RasGRP1⁻/⁻ mice are chronically immunodeficient and that forces driving wild-type T cell expansion in RasGRP1⁻/⁻ hosts could be a combination of self- and foreign-Ags.

RasGRP1⁻/⁻ CD4 T cells proliferate vigorously in chronically immunodeficient RAG-1⁻/⁻ mice

The previous experiments examined how wild-type T cells respond following transplant into RasGRP1⁻/⁻ hosts and therefore may not be reflective of how RasGRP1⁺/⁺ T cells react to environmental cues. To define how mutant T cells respond in these different settings, CFSE-labeled RasGRP1⁻/⁻ T cells (Thy1.2⁺) were i.v. injected into normal B6 (Thy1.1⁺), irradiated B6 (Thy1.1⁺), and RAG-1⁻/⁻ animals (Fig. 4A). Strikingly, the majority of RasGRP1⁻/⁻ CD4 T cells recovered from either normal or irradiated B6 animals were not recruited into cell cycle (63 and 65%, respectively) while the large fraction of RasGRP1⁻/⁻ CD8 T cells isolated from irradiated recipients were proliferating slowly (87%). By contrast, RasGRP1⁻/⁻ CD4 and CD8 T cells transplanted into RAG-1⁻/⁻ hosts proliferated vigorously. Furthermore, RasGRP1⁻/⁻ T cells, particularly the CD4 T cells, recovered from RAG-1⁻/⁻ recipients strongly down-regulated CD62L expression whereas those placed in irradiated B6 hosts resembled the surface phenotype before adoptive transfer (Figs. 4B and 1C). Next, we addressed whether the residence of RasGRP1⁻/⁻ T cells in different hosts influenced effector function (Fig. 4C). Upon TCR stimulation, RasGRP1⁻/⁻ T cells recovered from RAG-1⁻/⁻ hosts possessed an increased frequency of cells capable of producing the proinflammatory cytokines TNF-α and IFN-γ vs those residing in irradiated wild-type recipients. In summation, these studies demonstrate that chronically immunodeficient hosts are capable of inducing RasGRP1⁻/⁻ T cells to proliferate rapidly and boost effector function.

RasGRP1⁻/⁻ mice generate poor pathogen-specific T cell responses

To test the hypothesis that RasGRP1⁻/⁻ mice are immunodeficient, wild-type and mutant mice were infected with a novel recombinant strain of L. monocytogenes (rLM-SIY) expressing a MHC class I Kb-restricted peptide SIYRYYGL (SIY), an agonist for the 2C TCR (24). One week postinfection, splenocytes were stimulated with either the endogeneous MHC class II-restricted peptide LLO₁₉₀₋₂₀₁ (listeriolysin O (LLO); Fig. 5A) or SIY (Fig. 5B) and Ag-specific T cell responses were monitored by IFN-γ production using intracellular flow cytometry. As an additional control, splenocytes were also cultured on anti-TCR Ab-coated plates to test for the capacity to produce IFN-γ. Strikingly, RasGRP1⁻/⁻ mice mounted a barely detectable immune response toward the LLO peptide (Fig. 5C). The fact that RasGRP1⁻/⁻ CD4 T cells can respond to anti-TCR Abs suggests that the weak response by RasGRP1⁻/⁻ mice is the result of a failure to generate LLO-reactive T cells rather than to secrete IFN-γ. By contrast, RasGRP1⁻/⁻ CD8 T cells generate a strong anti-SIY response that is modestly reduced in Ag-specific T cell numbers as compared with wild type (Fig. 5C). In addition, we tested the function of RasGRP1⁻/⁻ CD8 T cell effectors in a standard ⁵¹Cr-release assay and found that these cells displayed cytotoxicity similar to their wild-type counterparts (Fig. 5D). These rLM-SIY infection studies suggest that RasGRP1 is particularly critical for generating MHC class II-restricted immune responses.

**FIGURE 5.** RasGRP1⁻/⁻ mice mount a poor MHC class II-restricted LLO response upon infection with L. monocytogenes. Wild-type and RasGRP1⁻/⁻ animals were infected i.v. with rLM-SIY. Spleens of infected mice were harvested 7 days postinfection and assayed for immune responses toward the immunodominant MHC class II-restricted peptide LLO₁₉₀₋₂₀₁ (A) or the MHC class I-restricted peptide SIY (B) by measuring IFN-γ production using intracellular flow cytometry. Numbers within the plot reflects the frequency of CD8 or CD4 T cells responding to a particular condition. C, RasGRP1⁻/⁻ mice possess reduced numbers of anti-LLO-reactive CD4 T cells. Error bars, SD. D, RasGRP1⁻/⁻ CD8 T cell effectors display potent cytotoxic activity. Various numbers of splenic CD8 T cells from wild-type and mutant mice were incubated with a ⁵¹Cr-labeled EL-4 target.
FIGURE 6. RasGRP1−/− animals generate a weak T cell response toward LCMV and exhibit delayed viral clearance. Wild-type and mutant mice were infected i.p. with the LCMV. At day 8 postinfection, splenocytes were cultured in either medium alone (no peptide), stimulated with the indicated immunodominant viral peptide or placed in a well coated with anti-TCR Ab (αTCR). Anti-LCMV T cells were enumerated by IFN-γ production by gating on either CD4 (A) or CD8 (B) T cells. Numbers within the plot reflects the frequency of CD8 or CD4 T cells responding to a particular condition. C. RasGRP1−/− animals possess greatly reduced numbers of Ag-specific T cells. Error bars represent the SD. D. At day 8 postinfection, LCMV can still be detected in the spleens of some RasGRP1−/− animals. Dashed line indicates the approximate detection level of the assay.

To address the possibility that the importance of RasGRP1 on mounting MHC class II-restricted immune responses may be restricted to the LLO peptide or bacterial infection, we used LCMV to infect wild-type and RasGRP1−/− mice. Eight days postinfection with a sublethal dose of LCMV, Ag-specific T cells were enumerated to viral immunodominant MHC class II-restricted GP61–80 (Fig. 6C) and MHC class I-restricted peptides (GP33–41, GP34–43, GP276–286, NP205–213, and NP396–404; Fig. 6D). Similar to the bacterial infection, RasGRP1−/− mice generated a weak MHC class II-restricted response although this time a reduced fraction of mutant CD4 T cells produced IFN-γ upon TCR stimulation (3.6 vs 13.0%). However, in contrast to rLM-SIY results, RasGRP1−/− mice mounted weak immune responses toward all five MHC class I-restricted viral peptides analyzed despite the fact that a similar proportion of wild-type vs mutant CD8 T cells can secrete IFN-γ upon TCR stimulation (48%). The fact that total T cell numbers were also sharply reduced in RasGRP1−/− animals makes the drop in Ag-specific T cell numbers even more dramatic (Fig. 6C). To evaluate whether the diminished T cell responses were physiologically relevant, splenic tissue, harvested 8 days postinfection, was screened for virus using plaque-forming assays (Fig. 6D). Indeed, 3 of 4 RasGRP1−/− spleens in this experiment (9 of 13 total) contained readily detectable virus at this late period postinfection, at a time point when virus is undetectable among wild-type mice. In conclusion, the weakened immune responses by RasGRP1−/− mice is associated with delayed viral clearance.

The ability of RasGRP1−/− mice to respond strongly to the SIY peptide but only weakly to immunodominant LCMV peptides led us to speculate whether the differential responses were intrinsic to the peptides or the pathogen. To resolve this issue, wild-type and RasGRP1−/− mice were infected with two other rLMs, either rLM-OVA or rLM-GP33, and the frequency of MHC class I-restricted anti-OVA257–264 and anti-GP33–41-specific T cells assessed 7 days postinfection. In stark contrast to the SIY peptide, RasGRP1−/− mice mounted weak, barely detectable responses toward both OVA257–264 and GP33–41 MHC class I-restricted peptides (our unpublished observations). These findings that RasGRP1−/− mice can respond to a limited set of immunodominant peptides suggest that its resident T cells may possess an altered TCR repertoire. To look for differences in the TCR repertoire between wild-type and RasGRP1−/− mice, Vβ TCR chain usages were determined by staining splenocytes with various anti-Vβ TCR chain-specific Abs in conjunction with Abs specific for CD4, CD8, and the constant region of the TCRβ chain (Fig. 7). Although we observed differences among the Vβ TCRs used by both RasGRP1−/− CD4 and CD8 T cells, the CD4 T cell population exhibited fewer statistically significant changes, due in part to a large deviation between RasGRP1−/− mice. It is not clear whether the TCR repertoire changes in RasGRP1−/− mice results from aberrant T cell development, homeostatic strain, or a mixture of both processes. Moreover, the possibility exists that CD4 T cell immune activation, resulting in persistent rounds of cellular proliferation and death, could contribute to great variability exhibited by RasGRP1−/− CD4 T cell TCR repertoire. Collectively, these studies raise the prospect that changes in the TCR repertoire in...
RasGRP1\(^{-/-}\) mice could be responsible for the impaired generation of pathogen-specific T cells.

**Failure of RasGRP1\(^{-/-}\) mice to generate pathogen-specific T cells is the result of RasGRP1 deficiency in thymocytes and/or T cells**

Because the engineered mutation in RasGRP1 results in a systemic loss of RasGRP1 function (14), it is possible that RasGRP1 deficiency in another cell type, besides T cells, may contribute to defective pathogen-specific responses. To investigate whether the innate immune system within RasGRP1\(^{-/-}\) mice is capable of nurturing T cell responses, mutant mice (Ly5.2\(^{+}\)) were infected with purified wild-type T cells (Ly5.1\(^{+}\)) and found the next day with either rLM-SIY or LCMV (Fig. 8A). Seven days post-rLM-SIY infection, a sizable proportion (7.1%) of the wild-type CD4 T cells were LLO reactive whereas few RasGRP1\(^{-/-}\) CD4 T cells produced IFN-\(\gamma\) upon peptide stimulation (Fig. 8B). Interestingly, RasGRP1\(^{-/-}\) CD8 T cells (Ly5.1\(^{+}\)) mounted a weaker anti-SIY response in the presence of wild-type T cells (Figs. 8B and 5B), implying that they may not compete well for Ag. These findings suggest that the innate immune system in RasGRP1\(^{-/-}\) mice is not to blame for the impaired generation of rLM-specific T cells.

To determine whether the wild-type T cells could also respond in mutant mice after viral infection, we performed an analogous experiment except this time we infected mutant mice (Ly5.2\(^{+}\)) with either rLM-SIY or LCMV. Splenocytes were recovered from animals after infection with either rLM-SIY (B) or LCMV (C), stimulated with the indicated peptides in vitro, and IFN-\(\gamma\) production measured by flow cytometry. Data were electronically gated on either CD4 or CD8 and the frequency of responding wild-type (Ly5.1\(^{+}\); right) or RasGRP1\(^{-/-}\) T cells (Ly5.1\(^{+}\); left) is indicated within the density plot.

**Discussion**

The prime directives of thymocyte development are to generate a TCR repertoire that is self-restricted, self-tolerant, and diverse, enabling responses toward a vast array of foreign peptides associated with self-MHC molecules. Because the generation of the TCR repertoire is dependent on TCR signaling, mutations affecting signaling molecules downstream of the TCR may have deleterious effects on both T cell function and TCR repertoire. In this study, we report the consequences of RasGRP1 deficiency and reduced TCR-induced Ras signaling on peripheral T cell homeostasis and T cell immunity.

A recently described mouse strain called RasGRP1\(^{lag}\) suffers from massive lymphoproliferation and an autoimmune syndrome sharing similarities with SLE (18). Although young mice appeared normal, older RasGRP1\(^{lag}\) mice developed massive lymphoproliferation, displaying splenomegaly and lymphadenopathy, with an excess of 10-fold larger lymph node size and cell numbers as compared with age-matched controls (18). By 5–8 mo of age, RasGRP1\(^{lag}\) mice were found to be so anorexic and lethargic that it necessitated euthanasia (18). Although we observed RasGRP1\(^{-/-}\) mice that developed substantial splenomegaly and lymphadenopathy (our unpublished observations), the penetration of this phenotype disappeared after successive backcrossing of the targeted RasGRP1 mutation to the B6 background. Our B6 backcrossed RasGRP1\(^{-/-}\) mice remain T cell lymphopenic and appear healthy until at least 1 year of age. However, despite the absence of massive lymphoproliferation, these RasGRP1\(^{-/-}\) mice do possess elevated levels of serum autoantibodies (our unpublished observations). Because autoimmune disease often requires a complex mixture of genetics and environmental factors (25, 26), it is perhaps not surprising that a change in genetic background may be responsible for the contradictions between our findings and those previously reported (18). Moreover, a recent study has found that SLE can simply develop from a hybrid 129/B6 background rather than targeted gene disruption (27). Therefore, we suspect that genetic modifiers from the 129/SvJ mouse strain may synergize with
RasGRP1 deficiency to cause massive lymphoproliferation and exacerbate autoimmune disease.

It has been proposed that RasGRP1-deficient thymocytes capable of maturing into SP thymocytes need to express more strongly self-reactive TCRs to overcome their signaling deficits (16–18). Because central tolerance does not appear to be affected by RasGRP1 deficiency (16), it has led us to hypothesize that self-reactivity of TCRs mediating positive selection of RasGRP1−/− double-positive thymocytes (DP) must bridge the boundary between positive and negative selection. The question of why immune activation selectively affects the CD4 T cell lineage in RasGRP1−/− mice is unknown. Because CD4 T cell development has been proposed to be more highly dependent on RasGRP1/ERK signaling (17, 28), RasGRP1 deficiency may affect thymic ontogeny by selecting more strongly self-reactive CD4 than CD8 SP thymocytes. Alternatively, RasGRP1-dependent mechanisms preserving peripheral tolerance or a relentless homeostatic strain may preferentially induce the activation of RasGRP1−/− CD4 T cells. However, because RasGRP1−/− T cells are severely hyporesponsive as compared with wild-type cells (16), their autoimmune potential may be counterbalanced by their inefficient TCR signaling and reduced proliferation upon Ag encounter.

Homeostatic mechanisms that function to regulate peripheral T cell numbers may be basis for the association between autoimmunity and T cell lymphopenia observed in both animals and humans (4, 25). Our studies of the homeostatic mechanisms operating in RasGRP1−/− mice suggest that both self- and foreign-Ags could be driving T cell proliferation (Fig. 3). However, because the RasGRP1 mutation was made in 129/SvJ embryonic stem cells (14), it is plausible that 129/SvJ-derived alloantigens may be responsible for some donor wild-type B6 T cell proliferation observed after their transfer into RasGRP1−/− mice (Fig. 3). Although the RasGRP1−/− mice used in this study have been bred at least seven generations onto the B6 background, this mouse line may still contain a significant amount of 129/SvJ DNA that is likely closely linked to the targeted locus. To identify the forces driving the cell cycling of RasGRP1−/− CD4 T cells in vivo (Fig. 1E), we initiated a series of adoptive transfer experiments using RasGRP1−/− donor T cells. Notably, it had been hypothesized that RasGRP1 deficiency allows autoreactive T cells to escape the thymus, proliferate upon encounter with peripheral self-Ags, and initiate autoimmunity (18). However, the failure of the majority of RasGRP1−/− CD4 T cells to proliferate after placement in wild-type hosts, either unmanipulated recipients or ones made lymphopenic through irradiation, suggests that self-Ags may not be responsible for their expansion (Fig. 4). By contrast, the observation that a subset of RasGRP1−/− CD4 T cells can undergo massive expansion in RAG-deficient hosts suggests that foreign Ags could be stimulating CD4 T cell proliferation in RasGRP1−/− animals (Fig. 4). Moreover, foreign Ags may be ideally suited to provoke weakly responsive RasGRP1-deficient CD4 T cells to proliferate vigorously because they can simultaneously act as a direct TCR stimulus, an activator of APCs and an inducer of inflammatory cytokine production. Therefore, these studies demonstrate that the environment within irradiated wild-type mice, possessing increased availability to both self-Ags and cytokines, is insufficient to recruit most RasGRP1−/− CD4 T cells into cell cycle.

The observation that RasGRP1−/− mice exhibit diminished T cell responses and delayed pathogen clearance suggests that they could be prone to developing chronic infections. Notably, RasGRP1−/− CD4 T cells share some attributes with functionally exhausted memory T cells found in both mice and humans during chronic infections (8, 9). First, RasGRP1−/− CD4 T cells possess markers of acute activation and T cell memory like exhausted T cells (Fig. 1C). Second, RasGRP1−/− CD4 T cells seem to have limited self-renewal capacity because they expand poorly in irradiated wild-type recipients, a cellular environment where the availability of the common γ-chain-linked cytokines IL-7 and IL-15 is increased (Fig. 4). RasGRP1−/− CD4 T cells also have reduced IL-7R expression (Fig. 1C). Third, RasGRP1−/− CD4 T cells strongly express PD-1, an inhibitory receptor that is coupled with exhausted viral-specific CD8 T cells in both mice and humans (11, 12), as well as its ligand PD-L1 (Fig. 1D). In contrast to the CD4 T cell phenotype, RasGRP1−/− CD8 T cells do not possess an exhausted phenotype because they do not exhibit signs of acute activation (CD69low, CD62Lhigh, CD127high; Fig. 1C), fail to express elevated levels of PD-1 (Fig. 1E) and can mount a significant anti-SIY T cell response (Fig. 5, B–D). A complication of housing chronically activated CD4 T cells is that it could promote autoimmunity in RasGRP1−/− mice, perhaps through elevated FasL expression, inducing nonspecific cell death, or proinflammatory TNF-α production (Figs. 1C and 4C). Therefore, T cell immuno-deficiency could predispose RasGRP1−/− mice to both chronic infections and autoimmunity.

The failure of RasGRP1−/− mice to generate normal numbers of Ag-specific CD4 and CD8 T cells after bacterial and viral infection likely results from a mixture of direct (T cell hyporesponsiveness) and indirect (altered thymic development and T cell homeostasis) influences of RasGRP1 deficiency. Decreased T cell responsiveness would be predicted to reduce the probability that a given T cell undergoes Ag-induced developmental programming whereas changes to T cell development and peripheral T cell homeostasis could alter the TCR repertoire and T cell differentiation. Notably, studies on RasGRP1−/− CD8 T cells have found that RasGRP1 regulates homeostatic proliferation (16), TCR-signaling thresholds, and augments cytokine production (J. Priatel, X. Chen, L. Zenzewicz, H. Shen, J. Coughlin, J. Stone, and H. Teh. manuscript in preparation). Because the precursor frequency of Ag-specific T cells is a critical parameter for the generation of effector and memory T cells (29, 30), we assessed Vβ TCR usage among splenic T cells from naïve (uninfected) mice to look for alterations in the TCR repertoire between normal and mutant mice. Although our results demonstrate that the TCR repertoire of RasGRP1−/− mice is significantly different, they do not provide a measure of TCR diversity. Moreover, the CD44high surface phenotype of RasGRP1−/− T cells (Fig. 1C) suggests the possibility that these cells may be derived from a considerable amount of peripheral expansion, a phenomena thought to restrict the TCR repertoire because it could result from the selective outgrowth of cells expressing a given TCR. In addition, the exhausted phenotype of RasGRP1−/− CD4 T cells suggests that their state of T cell differentiation could also contribute to their inaction. Therefore, reduced generation of Ag-specific T cells by RasGRP1−/− mice could result from changes in T cell differentiation, T cell function, and/or T cell TCR repertoire.

The fact that numerous T cell lymphopenic animals and humans exhibit T cell activation argues that an “empty” T cell compartment and changes to T cell homeostasis play a major role in the phenotype observed in RasGRP1−/− mice rather than being solely attributable to RasGRP1 loss in peripheral T cells. Interestingly, T cell lymphopenia is also often associated with T cell hyporesponsiveness and autoimmunity (25) and as a consequence, it raises the question of what are common denominators between these phenomena. The knowledge that TCR signaling is critically important for both T cell development and T cell survival has provided insight into the pairing of these occurrences (1, 31). Moreover, mutations affecting TCR signaling may cause T cell activation by...
IMMUNE SYSTEM DYSFUNCTION IN MICE LACKING RasGRP1

...determines the source of antigens that drive host-specific T cell expansion. J. Immunol. 174: 3158–3163.