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*J Immunol* 2006; 177:1470-1480; doi: 10.4049/jimmunol.177.3.1470
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RasGRP1 Transmits Prodifferentiation TCR Signaling That Is Crucial for CD4 T Cell Development

John J. Priatel, Xiaoxi Chen, Salim Dhanji, Ninan Abraham, and Hung-Sia Teh

TCR signaling plays a governing role in both the survival and differentiation of bipotent double-positive thymocytes into the CD4+ and CD8+ single-positive T cell lineages. A central mediator of this developmental program is the small GTPase Ras, emitting cytoplasmic signals through downstream MAPK pathways and eventually affecting gene expression. TCR signal transduction orchestrates the activation of Ras by integrating at least two Ras-guanyl nucleotide exchange factors, RasGRP1 and Sos. In this study, we have characterized the relationship between RasGRP1 function and its potential roles in promoting ERK activity, cell survival, maturation, and lineage commitment. Investigations on RasGRP1−/− mice expressing a transgenic (Tg) MHC class II-restricted TCR revealed that the development of CD4 T cells expressing this Tg TCR is completely dependent on RasGRP1. Unexpectedly, a small number of functional CD8 single-positive thymocytes expressing the Tg MHC class II-restricted TCR exists in mutant mice. In addition, RasGRP1−/− double-positive thymocytes exhibit marked deficits in TCR-stimulated up-regulation of the positive selection marker CD69 and the antiapoptotic protein Bcl-2, whereas CD8 induction is unaffected. To evaluate the role of RasGRP1 in providing cellular survival signaling, we enforced Bcl-2 expression in RasGRP1−/− thymocytes. These studies demonstrate that RasGRP1 function cannot be fully complemented by Tg Bcl-2 expression. Therefore, we propose that RasGRP1 transmits differentiation signaling critically required for CD4 T cell development. The Journal of Immunology, 2006, 177: 1470–1480.

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Received for publication December 5, 2005. Accepted for publication May 5, 2006.

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1 This work was supported by Grant MOP-77547 from the Canadian Institutes of Health Research (to H.-S.T.). S.D. was supported by the Natural Sciences and Engineering Research Council of Canada and the Michael Smith Foundation for Health Research.

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3 Abbreviations used in this paper: DP, double positive; Tg, transgenic; SP, single positive; LAT, linker for activated T cell; PM, plasma membrane; PLCγ1, phospholipase Cγ1; HSA, heat-stable Ag; P-ERK, phosphorylated ERK; PCC, pigeon cytochrome c; 7-AAD, 7-aminoactinomycin D; IT, intrathymic injection; MFI, mean fluorescence intensity; ISP, immature SP thymocyte; DN, dominant negative.

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0022-1767/06/$02.00
MEK1 were not found to sway CD4 vs CD8 differentiation (11–13), mice bearing a hypersensitive mutant form of ERK2 gene favor the development of CD4 single-positive (SP) thymocytes (21). Further support for ERK signaling directing thymocyte fate has come from investigations using MEK1 inhibitors in combination with fetal thymic organ cultures (21–23). Therefore, the amplitude and/or kinetics of ERK activation may also be key in determining lineage decision (21–23). Moreover, ERK activity has recently been found to positively regulate TCR signaling by modifying the Lck tyrosine kinase and subsequently preventing its inactivation by the Src homology protein-1 phosphatase (24). Thus, it is possible that ERK may affect TCR signaling strength by lengthening the duration of Lck activity through a feedback-loop mechanism.

In thymocytes, at least two Ras-guanyl nucleotide exchange factors, the well-studied Sos (25) and the more recently identified RasGRP1 (26), mediate Ras activation. The action of these two Ras-guanyl nucleotide exchange factors is dependent on their relocation to membranes by two distinct mechanisms (27). TCR stimulation and subsequent LAT (linker for activated T cells) phosphorylation result in the Src homology 2-domain-mediated targeting of Grb2/Sos complex to the plasma membrane (PM). In contrast, the movement of RasGRP1 is dependent on phospho-LAT’s recruitment and activation of phospholipase C1 domain (PLC1). The action of PLC1, converting phosphatidylinositol 4,5 bisphosphate into inositol 3,4,5 triphosphate and diacylglycerol, results in membrane localization for RasGRP1 through its diacylglycerol-phosphate into inositol 3,4,5 triphosphate and diacylglycerol, results in membrane localization for RasGRP1 through its diacylglycerol-binding C1 domain (28, 29). The surprising finding that RasGRP1 is recruited to the Golgi membrane (30–32), activating Golgi-as-targeting of Grb2/Sos complex to the plasma membrane (PM). In contrast, the movement of RasGRP1 is dependent on phospho-LAT’s recruitment and activation of phospholipase C1 domain (PLC1). The action of PLC1, converting phosphatidylinositol 4,5 bisphosphate into inositol 3,4,5 triphosphate and diacylglycerol, results in membrane localization for RasGRP1 through its diacylglycerol-binding C1 domain (28, 29). The surprising finding that RasGRP1 is recruited to the Golgi membrane (30–32), activating Golgi-associated Ras, rather than at the PM adds further complexity to Ras signaling, potential cellular responses, and the induction of developmental programs. In addition to the differential localization, Ras activation also displays delayed or sustained kinetics at the Golgi relative to the PM. RasGRP1+/– mice bear a defect in late thymic selection, where mutant DP thymocytes undergo rearrangement of their TCRα genes and express normal levels of TCRβ/CDC but are poorly promoted to the mature SP lineages (26, 33). Previously, we have determined that RasGRP1-dependent and -independent mechanisms exist for ERK activation and positive selection of MHC class I-restricted TCRs (33). To further characterize the role of RasGRP1 in thymocyte maturation, examining its relative importance in CD4 T cell development and lineage commitment, we generated generated RasGRP1+/– mice that express a Tg MHC class II-specific TCR. Here, we show that in mutant mice, the development of CD4 T cells expressing a Tg MHC class II-restricted TCR is completely abrogated. Therefore, we find that RasGRP1 deficiency affects CD4 T cell development more profoundly than the development of CD8 T cells. From in vitro TCR-stimulation experiments, RasGRP1+/– DP thymocytes display a diminished capacity to up-regulate Bcl-2. Based on these findings, we hypothesized that an important role for RasGRP1 may be to maintain cell viability and thus enable the differentiation of DP into SP thymocytes. However, the failure of forced Bcl-2 expression to restore CD4 T cell development in RasGRP1-deficient mice suggests that the RasGRP1-Ras-MAPK pathway may be particularly important for imparting differentiation signals that are essential for CD4 T cell development.

Materials and Methods

Mice

RasGRP1-null breeder mice were provided by J. C. Stone (University of Alberta, Edmonton, Alberta, Canada) and bred onto C57BL/6J background at least seven generations before mating with TCR Tg mice. The TcrAND (34) and TcrH-Y (35) Tg mice were bred onto the C57BL/6 (H-2b) background. Breeders bearing the TcrAND transgene were purchased from Jackson ImmuNoResearch Laboratories. The Equ-Bcl-2-36 Tg mouse line expresses strongly in thymocytes and has been bred at least six generations onto the C57BL/6J background as described previously (36). The Equ-Bcl-2-36 Tg mouse line was provided by C. J. Ong (University of British Columbia, Vancouver, British Columbia, Canada). B6.PL-Thy1.1/Cy (Thy 1.1) mice were acquired from The Jackson Laboratory. 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 (RK1.5), CD8 (53-6.7), H-Y TCR (T3.70), TCRβ (H57-597), CD3e (2C11), heat-stable Ag (HSA; M1/69), CD127 (A7R34), CD69 (HI.2F3), CD44 (IM7), Thy1.1 (HIS51), and Thy1.2 (53-2.1) were purchased from eBioscience. Abs against CD5 (53-7.3), TCR Vα11 (RR8-1), TCR Vβ3 (KJ25), and Bcl-2 (3F11) were purchased from BD Biosciences. For phospho-ERK (P-ERK)-specific staining, cells were fixed in methanol-free 2% formaldehyde (Polysciences), washed, and made permeable by incubation in 90% methanol for 30 min on ice. Intracellular staining was conducted in FACS buffer (2% FCS in PBS) at room temperature for 30 min. Rabbit anti-P-ERK1/2 Ab (Cell Signaling Technology; catalog no. 9101) was detected with donkey anti-rabbit Ig F(ab)2/PE (Jackson ImmuNoResearch Laboratories; catalog no. 711-116-152). After blocking unbound binding sites of the secondary Ab with rabbit IgG, cells were subsequently incubated with anti-CD4-FITC and CD8-PerCP (BD Biosciences). Data were acquired using either a FACSscan or FACSCalibur and CellQuest software (BD Biosciences). Data were analyzed with CellQuest or FCSExpress software (www.fcspress.com).

Proliferation assays

SP thymocytes were identified by staining with anti-CD8-PE and anti-CD4-PE-Cy5 Abs and purified by cell sorting using a BD FACSvantage (BD Biosciences). For Ab stimulation, 96-well flat-bottom plates were coated with 10 μg/ml of either anti-CD3e (2C11) or anti-Vα11 (RR8-1) in PBS for 2 h at 37°C. After washing wells three times with PBS, 40,000 sorted cells were seeded per well in complete IMEM (Invitrogen Life Technologies) containing 20 U/ml IL-2. For peptide stimulation, 20,000 sorted cells were incubated with 5 × 10–5 irradiated C3H (H-2k) splenocytes in round-bottom 96-well plates with the indicated pigeon cytochrome c (PCC) concentration and IL-2 (20 U/ml). After 72 h, cultures were pulsed with 1 μCi of [3H]thymidine for 6 h to assess proliferation.

CTL assays

SP thymocytes were identified by staining with anti-CD8-PE and anti-CD4-PE-Cy5 Abs and purified by cell sorting using a BD FACSVantage. To generate effector T cells, purified SP CD4+CD8 and CD4−CD8− SP thymocytes were stimulated for 3–4 days on anti-AND TCR-Ab-coated dishes in the presence of exogenous IL-2 (20 U/ml). The H-2a-expressing CH12 lymphoma cells were 51Cr labeled and subsequently incubated with various numbers of effectors in the presence and absence of 10 μM PCC peptide. After 5 h, supernatants were assayed for released 51Cr and specific killing determined as described previously (37).

DP thymocyte stimulations

Tissue culture wells (24-well plate; Falcon) were coated with 0.5 ml of either PBS alone, 10 μg/ml anti-CD3e (2C11), or 10 μg/ml anti-CD3e plus 10 μg/ml anti-CD4 (GK1.5) Abs for 2 h at 37°C. After washing the wells three times with PBS, total thymocytes (2 × 107) were placed in culture in complete IMEM for 24 h, harvested, and stained with anti-CD4 (RM4-4), anti-CD8 (53-6.7), and 5 μg/ml 7-aminoactinomycin D (7-AAD; Calbiochem) for dead cell exclusion and various cell surface markers. For Bcl-2 expression, cells were fixed in 2% paraformaldehyde/PBS solution for 15 min, made permeable by treatment with 0.2% Tween 20/PBS for 15 min and stained with either anti-Bcl-2 or isotype-control Abs conjugated to PE (BD Biosciences) for 30 min on ice. Following two washes with PBS, sample data acquisition was performed on a FACSCalibur.

Cell signaling studies and immunoblotting

Cell sorting, using a FACSVantage, was used to purify DP thymocytes. For activation, sorted DP thymocytes (10^7/ml) were coated with 10 μg/ml human anti-CD3e Ab for 20 min on ice, washed, and suspended in prewarmed (37°C) medium containing 80 μg/ml of goat anti-hamster Ab (Jackson ImmunoResearch Laboratories) for the indicated periods. Cells were lysed in...
10 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, protease, and phosphatase inhibitors. Abs used for immunoblotting that recognize activated and total ERK, LAT, and Zap70 were purchased from Cell Signaling Technology.

### Intraguild injection (IT) experiments

DP thymocytes were electronically sorted exactly as described for coreceptor re-expression assay. Approximately 1 million sorted DP thymocytes (10-μl volume) were injected per thymic lobe of (nonirradiated) B6.PL-Thy1.1 (Thy1.1) mice. Two days postinjection, injected thymi were recovered and mashed into single-cell suspensions, at a concentration of 10⁵ cells/ml. To deplete host cells, thymocytes were labeled with 0.5 μg/ml anti-Thy1.1 Ab, washed, and incubated with (25 μg/ml) sheep anti-mouse-lg-coupled Dynabeads (catalog no. 110.31; Dynal Biotech). Following magnetic separation, the remaining cells were split into two portions: one aliquot for ex vivo coreceptor expression analyses of donor cells, whereas the second was pronase-treated for a coreceptor re-expression assay. Because binding of anti-CD4 mAb (GK1.5) may block the ability of pronase to subsequently strip surface CD4 molecules (38), we selected to assess CD4 and CD8 coreceptor re-expression by staining with Abs conjugated to fluorochromes distinct from those used for cell sorting and thus avoid potential residual fluorescence. All samples were stained with anti-CD4-allophycocyanin, anti-CD8-PE, and Thy1.2-FITC-conjugated Abs, using FACS Diva software (BD Biosciences) for both acquisition and analysis.

### Coreceptor re-expression assay

Thymic single-cell suspensions were stained with PE-Cy7-conjugated anti-CD4 (GK1.5) and APC-Cy7-conjugated anti-CD8 (53-6.7) Abs and electronically sorted using either a FACSVantage or FACSAria flow cytometer (BD Biosciences). To cleave off surface coreceptor expression, purified DP thymocytes were pronase-treated as described previously (38, 39). Briefly, thymocytes were extensively washed with PBS and incubated in 0.01% pronase (Calbiochem) and 100 μg/ml DNase I (Sigma-Aldrich) for 15 min at 37°C. The enzymatic reaction was quenched by washing with medium containing 10% FCS and placed in overnight cultures at 37°C in complete medium. After culture, thymocytes were stained with anti-CD4-APC, anti-CD8-PE, and anti-Thy1.2-FITC Abs, and data were acquired on a BD LSR II benchtop (BD Biosciences) cytometer using FACS Diva software (BD Biosciences). FCS used in these experiments had been depleted of endogenous steroids by pretreatment with 0.5% Norit A Charcoal (Sigma-Aldrich). To cleave off surface coreceptor expression, purified DP thymocytes were electronically sorted using either a FACSVantage or FACSAria flow cytometer (BD Biosciences). To cleave off surface coreceptor expression, purified DP thymocytes were electronically sorted using either a FACSVantage or FACSAria flow cytometer (BD Biosciences). FCS used in these experiments had been depleted of endogenous steroids by pretreatment with 0.5% Norit A Charcoal (Sigma-Aldrich).

### Results

CD4 SP thymocytes are critically dependent on RasGRP1 for their development

To address the role of RasGRP1 in the development of thymocytes expressing a MHC class II-restricted TCR, we crossed the AND TCR transgene (TcrAND) onto RasGRP1-null animals. In C57BL/6 (H-2b) mice, the AND TCR (Vα11, Vp3) selects DP thymocytes on the MHC class II molecule I-A<sup>b</sup> and directs the development of large numbers of CD4 SP thymocytes (34). Strikingly, analyses of thymic subpopulations, as visualized by labeling with anti-CD4 and anti-CD8 Abs, revealed that RasGRP1<sup>−/−</sup> TcrAND mice are virtually devoid of CD4 SP thymocytes (Fig. 1A). Associated with impaired thymic selection, RasGRP1<sup>−/−</sup> TcrAND DP thymocytes possess reduced TCR expression, as judged by staining with anti-Tg TCRs, anti-TCRβ or anti-CD3ε Abs, and appear slightly immature by HSA and CD5 levels (Fig. 1B). By contrast with the MHC class II-restricted AND TCR, our previous results with RasGRP1-deficient mice expressing MHC class I-restricted TCRs exhibited a more modest effect on CD8 T cell development (33). Moreover, mutant mice expressing the weakly selecting H-Y TCR resulted in about a 4-fold reduction in CD8 SP thymocyte number, whereas the recovery of CD8 SP thymocytes bearing the strongly selecting 2C TCR resembled wild-type animals. These results indicate that RasGRP1-independent mechanisms cannot compensate for the positive selection of CD4 SP thymocytes by the AND TCR despite being able to mediate the development of CD8 T cells expressing either the H-Y or 2C TCRs (33).

Decreased ERK activity in RasGRP1<sup>−/−</sup> TcrAND DP thymocytes is correlated with reductions in TCR and Bcl-2 expression

We have previously observed that RasGRP1-deficient DP thymocytes bearing MHC class I-restricted TCRs exhibit reduced levels of active ERK as compared with wild type (33). To examine the contribution of RasGRP1 on ERK activation under the defined TCR signaling conditions of a MHC class II TCR, we used a flow cytometric assay based on a phospho-specific ERK1/2 Ab as described previously (33). Thymocyte single-cell suspensions were rapidly generated in serum-free medium, fixed, and assayed for ERK activity (Fig. 2A). Analysis of RasGRP1<sup>−/−</sup> TcrAND DP thymocytes revealed a considerable reduction in the frequency of P-ERK1/2high cells (6.8 vs 20%, respectively; Fig. 2A). Next, we examined whether the level of active ERK correlated strongly with TCR up-regulation, an index of positive selection (Fig. 2B). Indeed, ERK activity corresponded well with TCR expression regardless of genotype. Wild-type TcrAND DP thymocytes exhibit a large fraction (36%) of P-ERK1/2high AND TCR<sup>high</sup> cells. Despite diminished positive selection in RasGRP1<sup>−/−</sup> TcrAND thymi, mutant DP thymocytes possess a smaller but significant fraction of P-ERK1/2high AND TCR<sup>high</sup> cells (7%). This observation is consistent with previous studies demonstrating a requirement for sustained ERK activity in T cell development (14–17). However, because our flow cytometric-based assay measures ERK activity during a narrow window in time, it is not possible to determine whether the reductions in ERK signaling are the result of decreased intensity, duration, or both.

Previous studies using MEK1 inhibitors have suggested that ERK signaling is important for inducing Bcl-2 expression during thymic selection (18, 19). To examine whether RasGRP1 facilitates T cell differentiation by influencing Bcl-2 levels, we assayed Bcl-2 expression in TcrAND DP thymocytes via intracellular flow cytometry (Fig. 2C). As noted earlier (40), wild-type TcrAND DP

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**FIGURE 1.** RasGRP1<sup>−/−</sup> mice expressing the MHC class II-restricted AND TCR lack CD4 SP thymocytes. A, Thymocytes from wild-type and mutant mice expressing MHC class II-restricted AND TCR were stained with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the dot plots. Thymocyte recovery from wild-type and RasGRP1-deficient TCR Tg animals were as follows: RasGRP1<sup>−/−</sup> TcrAND, 131.2 ± 20.1 × 10⁶ (n = 6); and RasGRP1<sup>−/−</sup> TcrAND, 95.4 ± 17.3 × 10⁶ (n = 7). B, Flow cytometric analyses of TCR and developmental marker expression displayed by wild-type (shaded, thin line) and mutant (bold-line) DP thymocytes.
thymocytes have elevated Bcl-2 levels as compared with wild-type thymocytes from a normal (non-TCR Tg) mouse, a likely consequence of the fact that every thymocyte in a TCR Tg mouse expresses a selectable TCR. By contrast, RasGRP1−/− TcrAND DP thymocytes have considerably lower Bcl-2 expression as compared with wild-type TcrAND DP thymocytes (33.4 vs 74.1 mean fluorescence intensity (MFI); Fig. 2C). These findings suggest that RasGRP1 may transduce survival signaling via a Bcl-2-dependent mechanism.

RasGRP1−/− DP thymocytes demonstrate defective P-ERK, CD69, and Bcl-2 up-regulation following TCR engagement

ERK activation is a critical event in T cell development, and its relative activity correlates with the level of positive selection (41). Our analyses of TCR Tg mice demonstrate that RasGRP1-deficient DP thymocytes possess reduced levels of active ERK in isolated cells ex vivo (Ref. 33; Fig. 2). However, these reductions may be an indirect effect, a consequence of impaired positive selection in mutant animals. Therefore, we sought to investigate the direct effect of RasGRP1 deficiency on TCR-mediated ERK activation by stimulating DP thymocytes in vitro. A previous study (26) has shown that RasGRP1−/− thymocytes do not appreciably activate ERK upon either PMA or TCR stimulation. However, since this result was obtained from total thymocytes, it raises a concern that the differential composition of wild-type and mutant thymocyte populations may be responsible for these observations. Therefore, for TCR signaling studies, we sorted DP thymocytes from normal (non-TCR Tg) wild-type and mutant thymi and found that they expressed similar amounts of TCR/CD3ε (Fig. 3, A and B). Purified RasGRP1+/+ and RasGRP1−/− DP thymocytes were subjected to anti-TCR cross-linking for the indicated times (Fig. 3C). Wild-type cells induced strong ERK1/2 phosphorylation after TCR stimulation, whereas mutant DP thymocytes exhibited only a weak signal indicating that optimal TCR-induced ERK1/2 phosphorylation in these cells is dependent on RasGRP1. However, Zap70 and LAT, molecules upstream of RasGRP1, exhibit near-normal phosphorylation following TCR stimulation (Fig. 3C). This finding indicates that TCR-induced ERK activation is diminished in RasGRP1−/− DP thymocytes, whereas more proximal TCR signaling appears unaffected.

To examine whether RasGRP1 has direct impact on the expression of various markers of positive selection, we took DP thymocytes from normal (non-TCR Tg) wild-type and mutant animals and cultured them in vitro for 1 day in the presence or absence of TCR stimulation (Fig. 3D). Previous studies have shown that TCR engagement of DP thymocytes can induce many aspects of positive selection in vitro (42, 43). For our studies, we used two types of stimulation, either anti-TCR alone or anti-TCR plus anti-CD4 Abs, to mimic conditions of weak or strong TCR signaling. Analysis of CD5 induction revealed that RasGRP1−/− DP thymocytes behave similarly to wild-type cells under both conditions tested (Fig. 3D). In contrast, mutant thymocytes do not efficiently up-regulate CD69 upon stimulation with either TCR agonist. To address the RasGRP1 function in supporting survival signaling, we assessed the ability of mutant thymocytes to elevate Bcl-2 expression following TCR engagement. Using anti-TCR Ab alone, RasGRP1−/− DP thymocytes failed to
up-regulate Bcl-2 significantly. However, when anti-TCR and anti-CD4 Abs are used in tandem, Bcl-2 can be induced in mutant thymocytes, although these levels are still lower than wild type (16.3 vs 35.8 MFI). These findings suggest that RasGRP1−/− DP thymocytes must receive strong TCR signaling for their positive selection and support the conclusion that RasGRP1 augments Bcl-2 expression.

Tg Bcl-2 expression fails to restore CD4 T cell development in RasGRP1-deficient mice

Positive selection entails the rescue of DP thymocytes from programmed cell death and is dependent on the appropriate level of TCR/self-MHC signaling to provide antiapoptotic cues, such as the induction of Bcl-2 (40, 44–46). Because our in vitro findings suggest that RasGRP1 promotes Bcl-2 levels (Fig. 3D), we sought to determine whether enforced Bcl-2 expression could rescue CD4 T cell development in RasGRP1-deficient mice. To test our hypothesis, we introduced the Eμ-Bcl-2 transgene into both wild-type and RasGRP1−/− TcrAND mice (Fig. 4).

In RasGRP1-null animals, transgene-driven Bcl-2 expression results in a sharp increase in CD4 SP thymocyte number (6.1 ± 2.4 × 10⁶ vs 0.31 ± 0.05 × 10⁶), although still considerably lower than wild type (46.0 ± 7.6 × 10⁶). Next, we examined whether Eμ-Bcl-2 RasGRP1−/− CD4 SP thymocytes display signs of differentiation. To address this question, we compared the level of TCR expression in Eμ-Bcl-2 RasGRP1−/− TcrAND DP and CD4 SP thymocytes relative to wild-type TcrAND CD4 SP thymocytes (Fig. 4C). These analyses revealed that Eμ-Bcl-2 RasGRP1−/− TcrAND CD4 SP thymocytes showed signs of positive selection because they had slightly elevated TCR expression vs DP thymocytes. This increase in TCR expression was observed regardless of whether cytometry was performed with anti-Tg TCRα-, anti-Tg TCRβ-, or anti-TCRβ constant region-specific Abs (Fig. 4C and data not shown). However, TCR expression by Eμ-Bcl-2 RasGRP1−/− TcrAND CD4 SP thymocytes is considerably lower than the level that is exhibited by wild-type TcrAND CD4 SP thymocytes. Corroborating these studies, the analysis of non-TCR Tg animals lacking RasGRP1 also revealed that Bcl-2 overexpression fails to restore CD4 SP thymocyte proportion or numbers (data not shown). Therefore, we conclude that the developmental arrest of RasGRP1−/− TcrAND DP thymocytes cannot be rescued by provision of Tg Bcl-2 expression.

In agreement with a previous study using lck−/−Bcl-2 TcrAND mice (40), the Eμ-Bcl-2 transgene has a dramatic effect on wild-type TcrAND thymi. These changes include an increase in total thymocyte number, the formation of massive numbers of AND TCR− CD8 SP thymocytes, and a modest augmentation of the CD4 SP thymocyte number (Fig. 4, A and B). Although Linette et al. (40) have suggested that these CD8 SP thymocytes are developmentally arrested, we have found that this population possesses characteristics of mature T cells—expressing high levels of AND TCR, bearing intermediate to low levels of HSA and proliferating when contacted by cognate Ag (data not shown). These findings suggest that limited survival signaling restricts the formation of CD8 SP thymocytes in wild-type TcrAND animals. However, the fact that enforced Bcl-2 expression results in only a modest increase in RasGRP1−/− TcrAND CD8 SP thymocyte number (8.0 ± 3.8 × 10⁶ vs 3.9 ± 0.8 × 10⁶) argues that their inefficient positive selection is not primarily due to deficient Bcl-2 expression. Therefore, we conclude that RasGRP1 is necessary for development of massive numbers of CD8 SP thymocytes seen in wild-type Eμ-Bcl-2 TcrAND mice and provides developmental cues that are independent of Bcl-2 expression.

RasGRP1-deficient TcrAND CD8 SP thymocytes resemble CD8 SP thymocytes that develop in RasGRP1−/− mice expressing the MHC class I-restricted H-Y TCR

In addition to the lack of CD4 SP thymocytes, another feature of RasGRP1−/− TcrAND thymus is a small population of CD8 SP thymocytes that expresses the CD8αβ coreceptor (Figs. 1A and 5A and data not shown). Uncannily, the thymic CD4/CD8 developmental profile of RasGRP1−/− TcrAND mice seems reminiscent of mutant (female) animals expressing the MHC class I-restricted H-Y TCR (Fig. 5A). However, in contradistinction to the comparison of wild-type and RasGRP1−/− TcrH-Y mice, there is no change in CD8 SP thymocyte frequency or cell number recovered from RasGRP1−/− TcrAND mice relative to wild type (Fig. 5B). To examine whether RasGRP1−/− TcrAND CD8 SP thymocytes might be a product of positive selection, we compared surface TCR expression and markers of differentiation between RasGRP1-deficient CD8 SP thymocytes expressing either the AND or H-Y TCR (Fig. 5C). Regardless of the TCR expressed, mutant CD8 SP thymocytes share an immature cell surface phenotype (HSAbright)

![FIGURE 4](http://www.jimmunol.org/accepted_files/figure.png)

**FIGURE 4.** Tg Bcl-2 expression fails to restore CD4 T cell development in RasGRP1−/− TcrAND mice. A. Thymic developmental profiles for wild-type TcrAND and RasGRP1-deficient TcrAND thymocytes bearing the Eμ-Bcl-2 transgene were ascertained by staining with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the density plot. B. Cell numbers present within thymic subpopulations among various mice shown in A. C. RasGRP1−/− TcrAND CD4 SP thymocytes (thin line) express intermediate TCR levels as compared with wild-type TcrAND CD4 SP thymocytes (shaded, thin line) and RasGRP1−/− TcrAND DP SP thymocytes (bold line). Dotted, gray line represents background autofluorescence staining.
FIGURE 5. RasGRP1<sup>−/−</sup> animals share a similar thymic developmental profile regardless of whether they express either the MHC class II-restricted or MHC class I-restricted TCR. A, Thymocytes from RasGRP1<sup>−/−</sup> TcrAND and TcrH-Y were stained with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the density plot. B, Cell numbers present within thymic subpopulations from both wild-type and RasGRP1-deficient TcrAND and TcrH-Y were determined. To facilitate comparison between various thymi, cell numbers present within thymic subpopulations were plotted in bar graph format as the percentage of cells relative to wild type. C, RasGRP1<sup>−/−</sup> TcrAND CD8 SP thymocytes express comparable levels of various maturation markers as RasGRP1<sup>−/−</sup> TcrH-Y CD8 SP thymocytes.

and possess low levels of TCR, CD5, and CD69. These observations suggest that RasGRP1<sup>−/−</sup> TcrAND CD8 SP thymocytes may be the product of weak and/or inefficient positive selection.

RasGRP1<sup>−/−</sup> TcrAND DP thymocytes give rise to a few CD8, but not CD4 SP thymocytes after intrathymic transfer

Some unusual features of RasGRP1<sup>−/−</sup> TcrAND CD8 SP thymocytes may raise doubt regarding their developmental origin. Their rarity and relative immaturity (HSA high, CD69 low, CD8 low) suggests the possibility that these cells may be immature SP thymocytes (ISPs), a transitional, developmental intermediate that exists between the dominant-negative (DN) and DP cell stages (47), rather than being progenies of DP thymocytes. To gain insight into the derivation of RasGRP1<sup>−/−</sup> TcrAND CD8 SP thymocytes, we used electronic cell sorting of DP thymocytes in combination with a previously described coreceptor re-expression assay (38). The ability of coreceptor re-expression assay to assess lineage commitment is based on the fact that developing DP thymocytes committing to either the CD4 or CD8 lineage terminate synthesis of the inappropriate coreceptor molecule. The coreceptor re-expression assay enables one to discern what coreceptor molecules are actively being synthesized by individual cells by first stripping thymocytes of pre-existing coreceptor molecules by treatment with a protease called "pronase," followed by culturing overnight at 37°C to enable cells to re-express coreceptor proteins and finally assessing their newly synthesized CD4/CD8 surface phenotype by flow cytometry.

To assess the developmental potential of RasGRP1<sup>−/−</sup> TcrAND DP thymocytes in vivo, we purified TcrAND DP thymocytes (Thy1.2<sup>+</sup>) by cell sorting (Cell purity: >98 for wild type, >99% RasGRP1<sup>−/−</sup>) before adoptively transferring them into B6 (Thy1.1<sup>+</sup>) host thymi (Fig. 6). Two days post-IT, recovered thymi were assessed for CD4/CD8/CD5/CD69<sup>+</sup> surface phenotype of donor cells either before (untreated) or after pronase treatment and overnight culture at 37°C (pronase/37°C). Under these in vivo conditions, RasGRP1<sup>−/−</sup> TcrAND DP thymocytes gave rise to CD8 SP thymocytes (4.4%) but no CD4 SP thymocytes (Fig. 6). Importantly, the frequency of RasGRP1<sup>−/−</sup> TcrAND CD8 SP thymocytes (3.75 ± 1.73%; n = 7) and CD4 SP thymocytes (0.02 ± 0.02%; n = 7) observed in seven independent DP thymocyte IT experiments are similar to what is observed in normal RasGRP1<sup>−/−</sup> TcrAND thymi (Fig. 1). By contrast, wild-type TcrAND DP thymocytes gave rise to larger frequencies of both CD4 (9.9 ± 1.7%; n = 5) and CD8 SP thymocytes.
and CD8 SP thymocytes (12.3 ± 3.2%; n = 5). The observed increase in CD8 lineage cells is in line with a recent report, which showed that weakly selecting (or nonselecting) neighboring thymocytes can shift some AND TCR-expressing thymocytes toward the CD8 lineage (48). True to form, the coreceptor re-expression assay revealed an increased proportion of lineage-committed cells and conversely a decreased proportion of DP thymocytes in both wild-type and mutant samples. More importantly, this assay indicated that RasGRP1 \(^{−/−}\) TcrAND DP thymocytes could give rise to only CD8 but not CD4 thymocytes. Oddly, a sizeable fraction of RasGRP1 \(^{−/−}\) TcrAND DP thymocytes failed to express either surface CD4 or CD8 molecules, particularly after pronase treatment, and it is unclear whether the lack of RasGRP1 signaling has either aborted their development or taken them toward a cryptic differentiation program with a CD4^-CD8^- destiny. Previously, the derivation of CD4^-CD8^- progeny has been associated with DP thymocytes cultured in medium alone (no signal) before their intrathymic transfer as compared with those treated with PMA/ionomycin or anti-TCR plus anti-CD2 Abs (39). In conclusion, our IT experiments indicate that RasGRP1 \(^{−/−}\) TcrAND DP thymocytes yield a similar percentage of CD8 SP thymocytes as observed in the thymi of unmanipulated RasGRP1 \(^{−/−}\) TcrAND mice. Although these findings do not exclude an alternate origin, our observations are consistent with the notion that CD8 SP thymocytes present in RasGRP1 \(^{−/−}\) TcrAND animals are derived from DP thymocytes.

**Mutant CD8 SP thymocytes express markers of positive selection, respond to a MHC class II-specific Ag, and differentiate into cytotoxic effectors**

We have previously observed that the formation of CD8 SP thymocytes is associated with an elevated level of active ERK relative to DP thymocytes (33). Therefore, we sought to determine whether TcrAND CD8 SP thymocytes possess a similarly heightened ERK activity directly ex vivo (Fig. 7A). Indeed, both wild-type and mutant TcrAND CD8 SP thymocytes exhibited an elevated level of ERK activity, typical of positive selection. However, the nonconformity of CD8 SP thymocytes expressing a MHC class II-restricted TCR combined with their immature surface phenotype poses the question of whether these cells have truly undergone the positive selection process. To address this issue, we next examined whether RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes exhibit two other events associated with the formation of SP thymocytes, IL-7R expression and elevated Bcl-2 levels. Somewhat surprisingly, RasGRP1-sufficient TcrAND CD8 SP thymocytes do not express detectable levels of IL-7R and have atypically low quantities of Bcl-2 (Fig. 7B). Apparently, the selective formation of CD4 SP thymocytes impairs CD8 SP thymocyte development in wild-type TcrAND mice (34, 48) and results in very few peripheral CD8^+ T cells (34). In contrast, RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes express IL-7R and display Bcl-2 amounts characteristic of SP thymocytes, suggesting that these cells have undergone positive selection. Again, RasGRP1 \(^{−/−}\) CD8 SP thymocytes expressing the AND TCR share quite similar attributes to those bearing H-Y TCRs. Therefore, in contradistinction to CD8 SP thymocytes from wild-type TcrAND mice, RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes bear hallmarks of mature SP thymocytes.

A more definitive measure of positive selection is acquisition of mature T cell function. Therefore, we sought to establish whether the CD8 SP thymocytes that develop in RasGRP1 \(^{−/−}\) TcrAND mice could proliferate upon TCR engagement. Upon placement into tissue culture wells coated with anti-TCR Ab (either anti-CD3e or anti-TCR V\(_\alpha\)11 Ab), mutant TcrAND CD8 SP thymocytes proliferated at least as strongly as wild-type TcrAND CD4 and CD8 SP thymocytes (Fig. 7C). Next, we tested the capacity of these subpopulations to react with the cognate Ag for the AND TCR, the PCC peptide (88-104) presented by MHC class II H-2Ek molecule (34). Equivalent numbers of responder T cells were incubated with various doses of peptide plus irradiated, H-2Ek-expressing stimulator splenocytes. In contrast to the proliferation assays based on plate-bound anti-TCR Abs, RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes proliferated less vigorously than wild-type TcrAND CD4 SP thymocytes, particularly at low peptide concentrations (Fig. 7D). A plausible reason for the poorer response to

**FIGURE 7.** CD8 SP thymocytes that develop in RasGRP1 \(^{−/−}\) TcrAND mice respond to MHC class II-specific peptide and differentiate in cytolytic effectors. A. TcrAND CD8 SP thymocytes possess elevated ERK activity. Numbers within histograms represent background subtracted MFIs. Shaded histograms represent staining observed with secondary alone. B. CD127 (IL-7Rα) and Bcl-2 histograms representing RasGRP1 \(^{+/+}\), RasGRP1 \(^{−/−}\), and isotype-control staining of CD8 SP thymocytes. C. Sorted RasGRP1 \(^{+/+}\) TcrAND CD4 SP, RasGRP1 \(^{−/−}\) TcrAND CD8 SP, and RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes were cultured on plates coated with either anti-CD3e or anti-V\(_{\alpha}11\) (AND TCR; clone RR8-1) Ab plus exogenous IL-2 as described in Materials and Methods. D. Sorted SP thymocytes, either RasGRP1 \(^{+/+}\) TcrAND CD4 SP, RasGRP1 \(^{+/+}\) TcrAND CD8 SP, or RasGRP1 \(^{−/−}\) TcrAND CD8 SP, were stimulated with various doses of PCC and exogenous of IL-2 as described in Materials and Methods. E. Using intracellular flow cytometry, RR8-1-activated RasGRP1 \(^{+/+}\) TcrAND CD4, RasGRP1 \(^{+/+}\) TcrAND CD8, and RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes were stained with anti-granzyme B Ab. F. RR8-1-activated RasGRP1 \(^{+/+}\) TcrAND CD4 SP and RasGRP1 \(^{−/−}\) TcrAND CD8 SP thymocytes were tested for their ability to kill the CH12 (H-Ek expressing) lymphoma either in the presence or absence of PCC. Assays were done in triplicate, and the error bars represent the SD. Data from one representative experiment of three are shown.
peptide stimulation is that TcrAND CD8 SP thymocytes lack the appropriate coreceptor for corecognition of the I-Ek MHC class II molecule.

The acquisition of effector function is another stringent evaluation of positive selection. To address this question, TcrAND SP thymocytes were activated with anti-TCR V\textsubscript{a,11} Ab and examined for hallmarks of CD8 T cell effector function, namely the expression of the cytotoxic-associated molecule granzyme B and cytolysis. In contrast to TcrAND CD4 SP thymocytes, TcrAND CD8 SP thymocytes, regardless of genotype, rapidly expressed high levels of granzyme B upon activation (Fig. 7E). Subsequently, we investigated whether activated TcrAND SP thymocytes could mediate peptide-specific cytolysis (Fig. 7F). Indeed, RasGRP1\textsuperscript{−/−} TcrAND CD8 SP thymocytes could kill \textsuperscript{51}Cr-labeled targets in a MHC class II-restricted (PCC)-dependent fashion. Wild-type TcrAND CD4 SP thymocytes were at least 10-fold less potent killers (mutant cells have a greater kill at 1:1 E:T ratio compared with 10:1 for wild type). Thus, despite expressing a relatively immature cell surface phenotype, RasGRP1\textsuperscript{−/−} TcrAND CD8 SP thymocytes can efficiently kill Ag-coated target cells and thus are functionally mature.

RasGRP1\textsuperscript{−/−} TcrAND animals possess significant numbers of peripheral AND TCR\textsuperscript{+} CD8\textsuperscript{+} T cells but very small numbers of AND TCR\textsuperscript{−} CD4 T cells

To investigate the consequences of altered thymic selection on the peripheral lymphoid compartments, we examined the splenic T cell composition in these animals (Fig. 8A). In RasGRP1\textsuperscript{−/−} TcrAND mice, there is a 35-fold reduction in AND TCR\textsuperscript{+} CD4 T cell numbers, whereas the recovery of AND TCR\textsuperscript{+} CD8 T cells is increased 1.8-fold relative to wild-type mice (splenic cell recovery; RasGRP1\textsuperscript{+/+} TcrAND, 98.7 ± 11.2 x 10\textsuperscript{6}, n = 3; RasGRP1\textsuperscript{−/−} TcrAND, 78.1 ± 7.1 x 10\textsuperscript{6}, n = 3). Curiously, mutant CD8 T cells express slightly lower levels of CD8α- and CD8β-chain (Fig. 8A and data not shown). It is also noteworthy that only 23% of peripheral CD4 T cells in RasGRP1\textsuperscript{−/−} mice express high levels of the AND TCR, suggesting that the majority of these cells exploit endogenous TCRα-chains for positive selection (Fig. 8B). By contrast, peripheral CD8 T cells from RasGRP1\textsuperscript{−/−} TcrAND mice exclusively express the AND TCR (Fig. 8B), consistent with the notion that these cells use the AND TCR for their development. Moreover, RasGRP1\textsuperscript{−/−} TcrAND CD8 T cells share a comparable cell surface phenotype to functionally mature female RasGRP1\textsuperscript{+/+} TcrH-Y CD8 T cells (Fig. 8C). These observations support the conclusion that the few CD8 T cells that develop in RasGRP1\textsuperscript{−/−} TcrAND mice are the product of the positive selection process mediated by the AND TCR.

**Discussion**

Experiments with TCR Tg mice have demonstrated that thymocyte cell fate is dependent on whether the TCR of a developing DP thymocyte recognizes self-MHC class I or -MHC class II molecules. The strength of signaling model of lineage commitment proposes that intensity and/or duration of a TCR signaling determines whether a developing DP thymocyte chooses a helper CD4+ or cytotoxic CD8+ T cell fate; stronger and/or sustained TCR signaling results in CD4 SP thymocytes, whereas weaker and/or transient signaling produces CD8 SP thymocytes. Defining the molecular mechanisms controlling this cell fate decision has been a central focus of many investigations. Roles of the Ras/MAPK signaling pathway in positive selection have been implied by studies using either pharmacologic inhibitors of MEK1/2 in vitro or Tg mice expressing copious amounts of either constitutively active or DN forms of Ras and downstream MAPK family members (49, 50). In addition, some studies have suggested that ERK signaling strength may also be deterministic in lineage commitment (21–23). Moreover, studies based on either transgene overexpression or pharmacologic inhibitors may be especially prone to pleiotropic effects. Thus, many questions remain on how surface TCR signal transduction connects to Ras activation, links up to MAPK signaling pathways, and contributes to signaling cues necessary for the survival and differentiation of DP thymocytes. Using RasGRP1-null mice, we have previously shown that RasGRP1-dependent and -independent mechanisms exist for ERK activation and positive selection of CD8 SP thymocytes (33). Because the formation of CD4 SP thymocytes is thought to be dependent on prolonged TCR signaling (51) and RasGRP1 induces Rac activation with sustained and/or delayed kinetics (30–32), we sought to investigate the relative importance of RasGRP1 in the development of thymocytes expressing a MHC class II-restricted TCR. Herein, we report that RasGRP1 is a crucial TCR signaling component for CD4 T cell development.

The surprising finding of CD8 SP thymocytes, instead of CD4 SP thymocytes, in RasGRP1\textsuperscript{−/−} TcrAND raised questions regarding their nature and origin. The CD8 dull-phenotype of these cells suggests the possibility that they may belong to the CD8\textsuperscript{low} lineage, because premature expression of the ε T cell lineage, because premature expression of the αβ TCR in mice bearing the TcrH-Y and TcrAND transgenes causes aberrant γδT cell development (52, 53). Moreover, male TcrH-Y (H-2\textsuperscript{b}) mice notably contain large numbers of mature H-Y TCR\textsuperscript{+} CD8\textsuperscript{low} and H-Y TCR\textsuperscript{+} DN T cells with γδ T cell-like properties (52, 53), whereas TcrAND transgene is normally associated with an abundance of mature AND TCR\textsuperscript{+} DN T cells possessing such traits (53). However, TcrH-Y (H-2\textsuperscript{b}) mice only develop CD8\textsuperscript{low} H-Y
TCR+ T cells when nominal Ag (male, H-2b) is present because they are absent in the positively selecting (female, H-2d) and non-selecting (male or female, H-2b) backgrounds (54). Because TcrAND mice on a H-2b background lack nominal Ag, RasGRP1−/− TcrAND CD8 SP thymocytes are unlikely to be yδ lineage "wannabe" cells analogous to those present in H-Y H-2b male mice. Another possibility regarding the origin of RasGRP1−/− TcrAND CD8 SP thymocytes is raised by their immature phenotype (HSAhigh, CD69low) that is often characteristic of ISPs rather than "true" (mature) CD8 SP thymocytes. In contrast to ISPs, RasGRP1−/− TcrAND CD8 SP thymocytes exhibit mature functional attributes and can be derived from RasGRP1−/− TcrAND DP thymocytes. Therefore, together with our intrathymic transfer experiments (Fig. 6), indicating that RasGRP1−/− TcrAND DP thymocytes can give rise to some CD8 but not CD4 SP thymocytes, it is reasonable to presume that CD8 SP thymocytes observed in RasGRP1−/− TcrAND mice are the product of inefficient positive selection and of the αβ lineage.

The fact that RasGRP1−/− DP thymocytes expressing a MHC class II-restricted TCR fail to develop into CD4 SP thymocytes but can form functional CD8 SP thymocytes suggests that CD4 T cell development may be much more dependent on RasGRP1 than the development of CD8 T cells. This conclusion is corroborated with our MHC class I-restricted TCR Tg studies (33), defining significant RasGRP1-independent mechanisms for the positive selection of CD8 SP thymocytes, and reduced ratio of CD4 vs CD8 SP thymocytes in both normal and TCR Tg RasGRP1−/− mice. The findings are also consistent with previous studies using thymocyte cultures in combination with MEK1 inhibitors to analyze the significance of the Ras/ERK pathway (21–23). These studies revealed that the formation of CD4 SP thymocytes is more dependent on ERK activation than CD8 SP thymocytes. Reinforcing these observations, a recent report on ERK-deficient mice has found that the elimination of ERK1 and ERK2 protein preferentially affects the CD4 lineage (55). Therefore, we propose that RasGRP1-deficiency reduces the propensity of TCR signaled-DP thymocytes to take the CD4 SP developmental path rather than directly influencing lineage commitment per se.

To examine whether RasGRP1 promotes CD4 T cell development by inducing Bcl-2 expression, we introduced the Eµ-Bcl-2 transgene into RasGRP1−/− TcrAND animals. In Eµ-Bcl-2 RasGRP1−/− TcrAND mice, we observed a substantive (20-fold) recovery of CD4 SP thymocytes vs RasGRP1−/− TcrAND animals lacking the Bcl-2 transgene. Because enforced Bcl-2 expression cannot transform DP thymocytes into SP thymocytes in the absence of MHC molecules (40), the prosurvival effect of Bcl-2 without TCR signaling is insufficient for the formation of SP thymocytes. Therefore, the increased presence of CD4 SP thymocytes in Eµ-Bcl-2 RasGRP1−/− TcrAND mice argue that RasGRP1 assists CD4 T cell development by providing survival signaling to DP thymocytes. However, the facts that the CD4 SP thymocyte number is still much less than wild type (7.5-fold decrease) and that the cells that develop have lower TCR expression indicates that RasGRP1 transmits differentiation signals independent of those necessary for Bcl-2 induction. Collectively, our data suggest that positive selection in RasGRP1−/− TcrAND is inefficient, with only a small proportion of DP thymocytes receiving TCR signaling strength necessary for differentiation.

The impaired T cell development in RasGRP1-deficient animals results in a large number of discernable alterations including decreased ERK activation, altered surface marker expression, and reduced Bcl-2 levels. Such changes may result either directly from RasGRP1-deficiency or indirectly, a consequence of delayed positive selection. To determine whether RasGRP1 function was unequivocally connected to some of these changes, we cultured DP thymocytes from non-TCR Tg mice in the presence or absence of anti-TCR Ab stimulation to mimic the positive selection process in vitro as described previously (42, 43). Although CD5 induction is not affected, RasGRP1−/− DP thymocytes fail to up-regulate the positive selection marker CD69. This observation is consistent with a previous study demonstrating that this TCR-mediated event is dependent on Ras activation (56). Our experiment also revealed that RasGRP1−/− DP thymocytes require conditions of stronger TCR signaling to elevate Bcl-2 levels. As a consequence, these data suggest that RasGRP1−/− DP thymocytes must be strongly self-reactive to overcome their signaling deficits and differentiate into SP thymocytes. Notably, a recent report has linked T cells that develop in the absence of RasGRP1 to autoimmune consequences in a novel mouse strain, bearing a spontaneous mutation in RasGRP1 (57).

The relative rarity of TcrAND CD8 SP thymocytes in the RasGRP1−/− mice may be attributable to a couple of factors. First, the lack of concordance between TCR/MHC specificity and coreceptor expression has been postulated to result in reduced avidity for MHC class II molecules, inefficient TCR signaling, and subsequent failure of such mismatched cells to develop. The facts that MHC class II-restricted CD8 T cells can be positively selected and function in CD4-deficient mice argues against this conclusion (6–8). However, although these experiments demonstrate that MHC class II-specific CD8 T cells can develop, their formation and maintenance may still be compromised. Second, RasGRP1 is necessary for the efficient development of CD8 SP thymocytes (33). It is possible that the down-regulation of CD4 and lack of RasGRP1 expression may collaborate to limit the formation of RasGRP1−/− TcrAND CD8 SP thymocytes. The inability of Tg Bcl-2 expression to greatly improve CD8 SP thymocyte numbers in RasGRP1−/− TcrAND mice suggests that their limited quantity is not the result of inadequate levels of Bcl-2 but rather inefficient TCR signaling required for CD8 T cell differentiation. Since T cells require optimal TCR and CD4/CD8 coreceptor stimulation for their differentiation and survival, it serves not only to facilitate self-restriction but also maintains concordance between MHC specificity, effector function, and the CD4/CD8 phenotype of T cells in normal mice.

It is noted that small numbers of MHC class II-restricted CD8 T cells have been observed in wild-type MHC class II-restricted TCR Tg animals (6, 58). One potential explanation for the origin of these cells is that they are a product of limiting niches for selection of CD4 SP thymocytes in wild-type TcrAND mice. Using the strength of signal model of lineage commitment, some DP thymocytes may receive weakened TCR signaling due to the intense competition for positively selecting ligands (self-peptides/self-MHC class II) and thus be diverted to the CD8 lineage. Previous studies have shown that the development of MHC class II-restricted CD8 T cells in these animals requires MHC class II molecules but is independent of endogenous TCRα-chains (6, 58). Although we cannot formally rule out the participation of endogenous TCRα-chains in the derivation of RasGRP1−/− TcrAND CD8 SP thymocytes, the fact that virtually all RasGRP1−/− TcrAND peripheral CD8 T cells express the AND TCR (Fig. 5B) implies that endogenous TCRα-chains are not critical for their development.

The emerging role of subcellular compartmentalization in cell signaling has impacted the Ras/MAPK pathway (59). Recent work has demonstrated that PLCγ1 selectively activates RasGRP1 on the Golgi and is therefore distinct from the Grb2/Sos pathway that activates Ras at the PM (30–32). Differential localization may serve to alter the duration of signaling and/or pair-activated Ras with a different subset of effectors. Pointedly, Golgi-activated Ras
has been found to have a delayed or sustained kinetics relative to PM-activated Ras (30–32). Such prolonged signaling by RasGRP1 may be critical to efficiently induce the expression of Th-POK (also known as eKrox), a Krüppel transcription factor family member that is both necessary and sufficient to direct thymocytes undergoing positive selection into CD4 lineage (60, 61). Collectively, our results position RasGRP1 and its product, Golgi-activated Ras, to play a crucial role in initiating the CD4 T cell differentiation program.

Acknowledgments
We thank Ken W. Harder and Mike Chow for valuable discussions; Soo-Jeet Teh for technical assistance; James C. Stone for RasGRP1-null breeder mice; Wilfred A. Jefferies for C3H (H-2b) mice; Christopher J. Ong for Eμ-bcl-2-36 Tg mice; Michael R. Gold for CH12 B cell line; Andy John- son and Jeff Duenas for cell-sorting at the University of British Columbia MultiUser Flow Cytometry Facility; and Bill Masin for animal husbandry.

Disclosures
The authors have no financial conflict of interest.

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