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*J Immunol* 2011; 187:151-163; Prepublished online 6 June 2011;
doi: 10.4049/jimmunol.1100178
http://www.jimmunol.org/content/187/1/151

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/06/06/jimmunol.1100178.DC1

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A Role for p120 RasGAP in Thymocyte Positive Selection and Survival of Naive T Cells

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Activation of the Ras small GTP-binding protein is necessary for normal T cell development and function. However, it is unknown which Ras GTPase-activating proteins (RasGAPs) inactivate Ras in T cells. We used a T cell-specific RASA1-deficient mouse model to investigate the role of the p120 RasGAP (RASA1) in T cells. Death of CD4+CD8+ double-positive thymocytes was increased in RASA1-deficient mice. Despite this finding, on an MHC class II-restricted TCR transgenic background, evidence was obtained for increased positive selection of thymocytes associated with augmented activation of the Ras–MAPK pathway. In the periphery, RASA1 was found to be dispensable as a regulator of Ras–MAPK activation and T cell functional responses induced by full agonist peptides. However, numbers of naive T cells were substantially reduced in RASA1-deficient mice. Loss of naive T cells in the absence of RASA1 could be attributed in part to impaired responsiveness to the IL-7 pro-survival cytokine. These findings reveal an important role for RASA1 as a regulator of double-positive survival and positive selection in the thymus as well as naive T cell survival in the periphery. The Journal of Immunology, 2011, 187: 151–163.

As is a small G protein tethered to the inner leaflet of the cell membrane that cycles between inactive GDP-bound and active GTP-bound states (1). In its GTP-bound state, Ras triggers activation of downstream signaling pathways, such as the MAPK pathway that regulates cell growth and differentiation (2). A plethora of studies have illustrated the importance of Ras and MAPK for T cell development and function. Thus, in the thymus, Ras–MAPK signal transduction is necessary for pre-TCR–induced transition of CD4+CD8− double-negative (DN) thymocytes into CD4+CD8+ double-positive (DP) thymocytes (3, 4). Furthermore, the Ras–MAPK pathway is essential for TCR-mediated positive selection of DP cells resulting in their maturation into CD4+ or CD8+ single-positive (SP) T cells (4–7). In peripheral T cells, TCR-induced activation of the Ras–MAPK pathway is necessary for T cell activation and differentiation (8, 9).

The mechanism by which the TCR activates Ras has been well studied and involves mobilization of the guanine–nucleotide exchange factors, mammalian son of sevenless and Ras guanine nucleotide releasing protein 1, to cell membranes (10–12). These guanine–nucleotide exchange factors activate Ras by ejecting GDP from the Ras guanine nucleotide-binding pocket, thereby permitting Ras to bind GTP. Inactivation of Ras involves Ras-mediated hydrolysis of GTP to GDP. However, Ras has only weak GTP hydrolase activity and, therefore, Ras GTPase-activating proteins (RasGAPs) are required for efficient inactivation of Ras (13, 14). Through physical interaction, RasGAPs increase the ability of Ras to hydrolyze GTP by several orders of magnitude.

At least 10 different RasGAPs have been identified in mammals (13). However, with the exception of neurofibromin 1 (NF1), which RasGAPs inactivate Ras in T cells is unknown. Thymus and spleens from T cell-deficient mice transplanted with bone marrow (BM) from nonconditional NF1-deficient mice contained increased numbers of thymocytes and T cells, respectively, compared with T cell-deficient mice transplanted with wild type BM, although ratios of thymocyte and T cell subsets were unchanged (15). Furthermore, quiescent T cells in mice that had received NF1-deficient BM showed increased constitutive levels of active MAPK, although MAPK activity was not greater or more prolonged in these cells after TCR engagement compared with wild type T cells. These findings indicate a role for NF1 as a constitutive rather than negative feedback regulator of Ras activation in the T cell lineage in addition to its function as a regulator of T cell homeostasis. However, it is unknown which RasGAP(s) regulate Ras activation once Ras-GTP levels have risen at key pre-TCR or TCR driven T cell developmental checkpoints or during the course of T cell activation.

Another prototypical RasGAP that is well expressed in T cells is p120 RasGAP (RASA1). Biochemical analyses have implicated RASA1 as regulator of Ras activation in T cells beforehand (16). However, nonconditional RASA1-deficient mice succumb at a relatively early point in embryonic development (17). Therefore, it has not been possible to perform BM adoptive transfer experiments to address definitively the importance of RASA1 in T cells. To examine this, we generated T cell-specific RASA1-deficient mice. Studies of these mice have revealed an important role for RASA1 as a regulator of thymocyte survival and positive selection in the proliferation of naive T cells in the periphery.

Materials and Methods

Mice

The generation of rasa1<sup>fl/fl</sup> mice with and without plck-cre and ub-ert2cre transgenes has been described previously (18). HY TCR transgenic (Tg) and AND TCR Tg rasa1<sup>fl/fl</sup> and rasa1<sup>fl/fl</sup> plck-cre mice were generated by cross-breeding with HY TCR Tg or AND TCR Tg mice (Taconic and JAX, respectively).
respectively). All mice are on a C57BL/6 (H-2b) genetic background. C57BL/6 and B10.BR (H-2d) mice were purchased from JAX. All mice were 2-3 mo of age at the time of experiments. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Tamoxifen administration
Tamoxifen (TM; Sigma) was administered i.p. (two injections of 80 mg/kg body weight on two consecutive days) to rasa<sup>ab</sup> ub-er23cre mouse and rasa<sup>ab</sup> control mice at 6-8 wk of age. Flow cytometric analysis of splenocytes from TM-injected mice was performed 2-3 mo after TM injection.

Flow cytometry
Subpopulations of thymocytes and splenocytes were enumerated by cell counting and flow cytometry using fluorochrome-conjugated CD4, CD8, CD25, CD69, CD44, CD462L, TCRβ, TCRγδ, HY TCR (T3.70), TCR Vβ3, TCR Vα11, heat stable Ag (HAS), NK1.1, CD45.1, and CD45.2 specific mAb (Becton Dickinson). For analysis of DN thymocytes, macrophages, dendritic cells, and epithelial cells were gated out of populations on the basis of light scatter properties, whereas NK cells and CD4<sup>+</sup>CD8<sup>+</sup> T cells and NKT cells were gated out on the basis of specific Ab staining. For AND and HY TCR Tg mice, analyses were performed upon Vβ3<sup>+</sup> and HY TCR<sup>+</sup> populations, respectively. In AND TCR Tg mice, stages A-D of positive selection were defined as indicated in Fig. 2A. In female HY TCR Tg mice, populations A-D were similarly defined, except that populations C and D were derived from a CD8 SP gate. In non-TCR Tg mice, populations A-D were defined on the basis of TCR versus CD69 expression as described (19, 20). CD8<sup>+</sup>intermediate SP cells in all types of mice were eliminated from CD8 SP gates on the basis of high levels of HSA expression. Cell viability was determined by staining with fluorochrome-coupled annexin V and 7-amino-actinomycin D (7AAD; Becton Dickinson). ERK activation was determined by intracellular staining using a fluorochrome-conjugated phosho-ERK specific mAb (Cell Signaling), as described previously with minor modifications (21). Cell staining was analyzed on a FACSCanto (Becton Dickinson).

Cell isolation
CD<sup>4</sup>+, CD<sup>8</sup>+, CD<sup>4</sup>CD<sup>44</sup><sup>-</sup> and CD<sup>8</sup>CD<sup>44</sup><sup>-</sup> T cells were isolated from lymph nodes (LNs) and spleens by negative selection using immunobeads (Miltenyi). Cell populations were >90% pure as determined by flow cytometry. Expression of RASA1 in unfractonated and fractionated cell populations was determined by Western blotting using the B4F8 mAb (Santa Cruz). Blots were stripped and reprobed with either β-actin or GAPDH Abs (Santa Cruz) to verify equivalent protein loading.

Bone marrow transfer experiments
BM from rasa<sup>ab</sup> pick-cle (CD45.2) mice and age- and sex-matched C57BL/6 wild type (CD45.1/CD45.2) mice was depleted of lineage-negative cells by complement-mediated lysis. Cells were then injected (5 × 10<sup>6</sup> cells each) into the tail veins of lethally irradiated (950 rad) C57BL/6 wild type recipient mice (CD45.1). After 6 wk, recipients were euthanized and spleens were analyzed by flow cytometry.

MAPK activation in peripheral T cells
For assessment of ERK activation in peripheral T cells, 1.5 × 10<sup>6</sup> CD<sup>4</sup><sup>+</sup>CD<sup>44</sup><sup>-</sup> T cells from AND TCR Tg mice were mixed with 1.5 × 10<sup>6</sup> irradiated splenic adherent cells from B10.BR mice that had been prepulsed with pigeon cytochrome c (PCC) peptide 88–104 (10 μg/ml) for 1 h. Cells were centrifuged, and cell pellets were kept on ice. The pellets were then transferred to 37°C for different times. ERK activation was determined by flow cytometry (see above) and Western blotting using a phospho-specific ERK Ab (Cell Signaling). Western blots were stripped and reprobed with an ERK Ab (Santa Cruz) to ascertain equivalent protein loading.

T cell cytokine synthesis and proliferation
For cytokine synthesis and proliferation assays, 2 × 10<sup>5</sup> CD<sup>4</sup>CD<sup>44</sup><sup>-</sup> AND T cells were stimulated with 1 × 10<sup>6</sup> irradiated B10.BR splenic adherent cells and different concentrations of PCC peptide or CD3 and CD28 mAb (1 μg/ml, eBioscience) in complete medium (RPMI 1640 containing FBS and antibiotics) in wells of 96-well U-bottom plates. Concentrations of IL-2 in culture supernatants were determined by ELISA after 48 h culture (R&D Systems). T cell proliferation was assessed by dilution of CFSE fluorescence after 48, 72, or 96 h of culture.

Thymocyte apoptosis
Thymocytes were seeded into wells of 96-well flat-bottom plates at 2 × 10<sup>5</sup> thymocytes per well in complete medium or RPMI 1640. Wells were precoated or not with CD3 and CD28 mAb by overnight incubation in PBS. Dexamethasone or staurosporine (Sigma) were added to wells at the indicated concentrations. After 20 h of culture, cells were harvested and viability was assessed by flow cytometry.

Activation induced cell death of peripheral T cells
CD<sup>4</sup>CD<sup>44</sup><sup>-</sup> AND T cells were stimulated with irradiated B10.BR splenic adherent cells and 10 μg/ml PCC peptide plus 100 ng/ml of recombinant IL-2 (R&D Systems), as indicated in cytokine synthesis and proliferation assays. After 96 h, cells were harvested, washed, and cultured in RPMI 1640 medium alone or complete medium supplemented or not with IL-2 (10 ng/ml). After an additional 48 h, cell viability was determined by flow cytometry.

Homeostatic proliferation
Five million CFSE-labeled CD<sup>8</sup>CD<sup>44</sup><sup>-</sup> T cells isolated from LN and spleen of rasa<sup>ab</sup> and rasa<sup>ab</sup> plck-cre mice (CD45.2) were injected into the tail veins of sublethally irradiated (500 rad) C57BL/6 mice (CD45.1). After 5 d, LN and spleen were harvested, and the extent of proliferation of donor T cells was determined by dilution of CFSE fluorescence.

Periperal T cell survival assays
Purified whole T cell populations from spleens of rasa<sup>ab</sup> and rasa<sup>ab</sup> plck-cre mice were cultured in wells of 24-well plastic plates (1 × 10<sup>6</sup> cells/well) in complete medium in the presence or absence of 5 ng/ml of recombinant IL-7 (R&D Systems). After 3 d of culture, cell viability was determined by flow cytometry.

Results
T cell development in T cell-specific RASA1-deficient mice
To delete RASA1 in the T cell lineage, rasa<sup>ab</sup> mice were crossed with pick-cre mice to generate rasa<sup>ab</sup> pick-cre mice (18). In these mice, Cre is expressed from the DN3 (CD44<sup>DC25</sup>) stage of thymocyte development onward (22). We first examined T cell development in these mice (Supplemental Fig. 1). In comparison with littermate control rasa<sup>ab</sup> mice, total numbers of DN and SP cells in thyms were largely unaltered. However, a modest reduction in DP number was observed. Although this finding was not statistically significant, the reduction in DP number was marked enough to cause a significant decrease in the DP/DN ratio in these mice (Supplemental Fig. 1C). Further analysis showed that an increased fraction of DP cells in RASA1-deficient mice stained positively for annexin V, indicating an increased susceptibility to apoptosis (data not shown). From the DP stage onward, we defined different successive stages of development based on expression levels of the ββ TCR and CD69 (19, 20). Each of population A (prepositive selection; CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>int</sup>CD69<sup>-</sup>), population B (postpositive selection; CD4<sup>+</sup>CD8<sup>-</sup>CD44<sup>+</sup>CD8<sup>+</sup>TCR<sup>+</sup>CD69<sup>-</sup>) and population C (postpositive selection; CD4<sup>+</sup>CD8<sup>-</sup>CD44<sup>+</sup>CD8<sup>+</sup>TCR<sup>+</sup>CD69<sup>-</sup>) thymocytes showed increased annexin and 7AAD staining in RASA1-deficient mice (Supplemental Fig. 1D). By contrast, increased apoptosis was not observed in DN3, DN4, or population D cells (mature; CD4<sup>+</sup>CD8<sup>-</sup>CD44<sup>+</sup>CD8<sup>+</sup>TCR<sup>+</sup>CD69<sup>-</sup>). Therefore, the reduced DP/DN ratio in RASA1-deficient thyms is most likely explained by the increased susceptibility of population A cells, which constitute the vast majority of DP cells, to apoptotic death rather than indicating impaired DN-to-DP transition.

T cell development in T cell-specific RASA1-deficient TCR transgenic mice
Because loss of RASA1 did not appear to affect DN to DP transition or positive selection, indicated by increased SP/DP ratios, we next examined T cell development in TCR Tg mice. It was...
reasoned that a role for RASA1 in these processes might be revealed in TCR Tg mice that would not be readily apparent in a polyclonal setting. To study the selection of MHC class II-restricted T cells, we generated rasa1fl/fl and rasa10fl plck-cre AND TCR Tg mice. The AND TCR has specificity for PCC peptide 88–104 in the context of the MHC class II molecule, I-Ee (23). In H-2a mice, AND TCR Tg DP cells are positively selected on the MHC class II molecule, I-Ae, and mature into CD4 SP T cells. Use of this model, therefore, affords an opportunity to examine positive selection on MHC class II at a clonal level.

Numbers of DN cells were not affected by the loss of RASA1 in AND TCR Tg mice. By contrast, substantial reductions in the number of DP and CD4 SP cells were observed (Fig. 1B, 1C). Annexin V staining of thymocyte subpopulations revealed that, similar to non-TCR Tg mice, DP cells but not DN cells showed increased apoptosis (data not shown and Fig. 2). Therefore, as before, the increased apoptosis of DP cells likely accounts for the decreased DP/DN ratio in the absence of RASA1 rather than indicating impaired DN/DN transition (Fig. 1C).

In contrast to the DP/DN ratio, the CD4 SP/DP ratio was increased in the absence of RASA1 (Fig. 1C). This finding could be taken as evidence for augmented positive selection in this model. Before further exploration, we wanted to ascertain that the decreased numbers of DP and CD4 SP cells was not a result of increased negative selection of AND TCR Tg cells. To address this, we compared the apoptotic response of wild type and RASA1-deficient thymocytes to increasing concentrations of CD3 mAB (directed to the TCR complex) plus mAB against the CD28 co-stimulatory receptor or to CD3 mAB alone (Fig. 3A). In wild type thymocytes, CD3 plus CD28 mAB but not CD3 mAB alone induced significant apoptosis in DP cells, as expected (24, 25). In contrast, neither CD3 plus CD28 mAB nor CD3 mAB alone induced significant apoptosis in wild type CD4 SP cells. The apoptotic responses of RASA1-deficient thymocytes to CD3 plus CD28 mAB and CD3 mAB alone were essentially identical to those of wild type thymocytes. In DP cells, CD3 plus CD28 mAB triggered the same extent of apoptosis over a range of mAB concentrations. Furthermore, sensitivity of DP cells to CD3 mAB alone or CD4 SP cells to CD3 plus CD28 mAB or CD3 mAB alone was not acquired with loss of RASA1 expression. Therefore, it is unlikely that the reduced number of DP and CD4 SP cells in RASA1-deficient AND TCR Tg thymi can be explained by increased negative selection. Interestingly, RASA1-deficient thymocytes also showed similar sensitivity to other established apoptosis-inducing agents or conditions in vitro including dexamethasone, staurosporine, and serum deprivation (Fig. 3B–D).

Another alternative interpretation for the increased CD4 SP/DP ratio in RASA1-deficient AND TCR Tg mice is that this ratio is artificially increased as a consequence of the increased death of DP cells. To address this interpretation, we again identified successive stages of T cell development from the DP stage onward. However, because of the relatively modest upregulation of CD69 on population B cells in AND TCR Tg mice, populations A and B were initially distinguished on the basis of CD4 and CD8 expression rather than TCR versus CD69 expression (Fig. 2A). Population C and D cells were identified within a CD4 SP gate and distinguished based on high or low expression of CD69 respectively (Fig. 2A). Examination of the expression levels of the TCR and HSA confirmed that populations A–D so defined in AND TCR Tg mice were analogous to the same populations in non-TCR Tg mice (data not shown).

Analysis of populations A–D in AND TCR Tg mice strongly supported the notion of increased positive selection. First, in population A cells, expression of CD69 was consistently increased in the absence of RASA1 (Fig. 2B). Upregulation of CD69 expression is characteristic of positive selection in the thymus (24, 25). Second, ratios of the postpositive-selection populations B and C to the prepositive-selection population A were both increased (Fig. 2C). These increases could not be accounted for by increased susceptibility of population A to apoptosis because populations A–C showed comparable increases in apoptosis in RASA1-deficient mice (Fig. 2D).

FIGURE 1. T cell development in T cell-specific RASA1-deficient AND TCR Tg mice. All experiments were performed with thymi of littermate rasa1fl/fl and rasa10fl plck-cre AND TCR Tg mice. A, Representative Western blot showing RASA1 expression in whole thymi. B, At left are shown representative two-color flow cytometry plots of CD44 and CD25 expression on CD4+CD8+ DN thymocytes. Numbers indicate the percent of cells in each quadrant. The bar graphs show the mean number plus 1 SEM of DN1 (CD44+CD25−), DN2 (CD44+CD25+), DN3 (CD44−CD25+), and DN4 (CD44−CD25−) cells in thymi (n = 5 mice of each genotype). C, Representative plots of CD4 and CD8 expression (left) and bar chart (middle) showing mean number plus 1 SEM of DN, DP, CD4, and CD8 SP cells in thymi (rasa10fl, n = 18; rasa10fl plck-cre, n = 20). The bar graphs depict indicated mean ratios plus 1 SEM of thymocyte subsets within each genotype. Statistical significance was determined using the Student two-sample t test. ***p < 0.001.
Increased positive selection in RASA1-deficient AND TCR Tg mice is consistent with the known function of RASA1 as a negative regulator of Ras-MAPK signal transduction. To confirm that RASA1 regulates this pathway during the course of positive selection, we examined the activation of ERK-MAPK in thymocyte subsets in freshly explanted thymi by phospho-flow cytometry (Fig. 4). In control AND TCR Tg thymi, distinct phospho-ERK-positive cells were difficult to identify by phospho-flow cytometry in any of populations A–D. By contrast, in RASA1-deficient AND TCR Tg thymi, phospho-ERK-positive cells were readily identified. Staining was highest in populations A and C and lowest in population D. Furthermore, background levels of ERK staining were consistently higher in all four thymocyte populations in RASA1-deficient mice, although not in peripheral T cells (discussed later). Increased activation of ERK, therefore, likely accounts for the increased positive selection in the absence of RASA1 (7).
To examine the selection of MHC class I-restricted T cells, we used HY TCR Tg mice. The HY TCR recognizes a male-specific HY peptide in the context of the MHC class I molecule, H-2Db (26). In female H-2b HY TCR Tg mice, HY TCR Tg DP cells are positively selected upon recognition of H-2Db and mature into CD8+ SP T cells.

Numbers of DN, DP, and SP cells in T cell-specific RASA1-deficient female HY TCR Tg mice were comparable to those observed in control female HY TCR Tg mice (Fig. 5B). A modest, though not statistically significant, reduction in DP number was noted that could be accounted for by increased apoptosis of population A cells, similar to non-TCR Tg and AND TCR Tg mice (Fig. 5C). However, the CD8 SP/DP ratio was not significantly changed. This finding indicates that in this particular MHC class I-restricted TCR Tg model, RASA1 does not have a role in positive selection.

In male HY TCR Tg mice, expression of the HY peptide on thymic stromal cells results in negative selection of HY TCR-expressing DP cells (26). Therefore, we examined the thymi of male RASA1-deficient HY TCR Tg mice to determine whether there was any influence of RASA1 loss on negative selection in this model (Fig. 5D). However, thymi from RASA1-deficient and control male HY TCR Tg mice showed the same reductions in total cellularity associated with loss of DP and SP cells compared with female HY TCR Tg mice (compare Fig. 5B). We conclude, therefore, that expression of RASA1 is not necessary for thymic negative selection in HY TCR Tg male mice. Moreover, there are no signs of autoimmune disease development in T cell-specific RASA1-deficient mice, which is consistent with the notion that negative selection is intact in these animals.

Peripheral T cells in T cell-specific RASA1-deficient mice

Despite normal numbers of CD4 and CD8 SP T cells being found in the thymi of T cell-specific, RASA1-deficient, non-TCR Tg mice, substantially reduced numbers of CD4 and CD8 T cells were found in peripheral lymphoid organs of these animals (Fig. 6B). (Figs. 6–9 show results from spleen; the same observations were made in LN throughout.) This loss of peripheral T cells was not caused by the plck-cre transgene itself, because rasa1+/crcre mice contained the same number of peripheral T cells as littermate rasa1+/mice (Supplemental Fig. 2). Furthermore, TM treatment of adult rasa1+/mice (to induce global

![FIGURE 3. Apoptosis sensitivity of RASA1-deficient thymocytes. Experiments were performed with thymocytes of littermate rasa1+/+ and rasa1+/+ plck-cre mice. A, Thymocytes were seeded into wells that had been precoated or not with different concentrations of CD3 mAb or CD3 plus CD28 mAb. After 20 h, the percentage of live annexin 7AAD+ DP or CD4 SP cells in cultures was determined by flow cytometry. Results were normalized to the percentage of live DP or CD4 SP cells harvested from wells that were not coated with mAbs. Graphs show the mean percentage of live cells ±1 SEM under the different stimulation conditions (n = 3 mice of each genotype). B and C, Experiments were conducted as in A except that dexamethasone (B) or staurosporine (C) were added to wells in different concentrations. Graphs show the mean percentage of live whole thymocytes ±1 SEM for each condition (n = 3 mice each genotype). D, Thymocytes were cultured in serum-free or complete medium for the indicated times. Cell viability was determined as in A. Results with serum-free medium were normalized to the percentage of viable cells cultured in complete medium for the same time. Bar graphs show the mean percentage of live whole thymocytes ±1 SEM at each time in serum free medium (n = 3 mice each genotype).](http://www.jimmunol.org/)

![FIGURE 4. ERK activation during positive selection in T cell-specific RASA1-deficient AND TCR Tg mice. Representative flow cytometric plots showing expression of phospho-ERK in the indicated thymocyte populations from littermate rasa1+/+ and rasa1+/+ plck-cre AND TCR Tg mice (see Fig. 2). Numbers represent the percent of phospho-ERK+ cells within each population. The same results were obtained in a repeated experiment. A–D, Populations A, B, C, and D, respectively.](http://www.jimmunol.org/)

![FIGURE 5.](http://www.jimmunol.org/)
deletion of RASA1) resulted in a loss of peripheral CD4 and CD8 T cells (Fig. 7). Therefore, expression of RASA1 in the T cell lineage is necessary to maintain normal numbers of T cells in the periphery. The importance of RASA1 in peripheral T cell homeostasis was further illustrated in competitive BM transfer experiments (Fig. 6C). In these experiments, equal numbers of lineage-negative BM cells from wild type and rasa1fl/fl plck-cre mice were injected into lethally irradiated wild type recipient mice. After 6 wk, spleens were harvested and the percentage of CD4 and CD8 T cells among splenocytes of donor wild type and rasa1fl/fl plck-cre BM origin was determined with the use of congenic CD45 markers. As shown, the percentage of CD4 and CD8 T cells among rasa1fl/fl plck-cre splenocytes was substantially less than the percentage of CD4 and CD8 T cells among wild type splenocytes.

We examined numbers of naive (CD44lo), central memory (CD44hiCD62Lhi), and effector memory (CD44hiCD62Llo) CD4 and CD8 T cells in T cell-specific RASA1-deficient non-TCR Tg mice (Fig. 6D). These studies revealed that the diminution of CD4 and CD8 T cell number was primarily accounted for by loss of naive T cells. A specific loss of naive T cells was also observed in rasa1fl/fl ub-ert2cre mice treated with TM (Fig. 7).

Analysis of peripheral T cells in RASA1-deficient AND TCR Tg mice revealed a similar diminution in the total number of CD4 T cells that was explained by a reduction in the number of naive CD4 T cells specifically (Fig. 8B–D). In addition, reduced numbers of CD8 T cells were found in the periphery of RASA1-deficient female HY TCR Tg mice compared with controls (Fig. 9B). In control female HY TCR Tg mice, the majority of peripheral CD8 T cells do not in fact express the clonotypic HY
TCR, defined by high-level expression of the T3.70 marker (Fig. 9C, right). This finding is not a result of inefficient allelic exclusion by the HY TCR during T cell development, because the vast majority of thymic CD8 SP T cells express high levels of T3.70 (Fig. 9C, left). Rather, as shown previously, HY TCR-expressing T cells are peculiarly unable to undergo homeostatic expansion in the periphery of female mice (27, 28). This finding is in contrast to HY TCR negative CD8 T cells and CD4 T cells, which are able to expand in the periphery and thus outgrow HY TCR T cells despite far fewer of these cells being generated in the thymus. Notably, numbers of peripheral HY TCR CD8 T cells are the same in female RASA1-deficient and control HY TCR Tg mice. Rather, there are fewer HY TCR-negative CD8 T cells in the periphery of the RASA1-deficient mice, which accounts entirely for the reduction in total CD8 T cell number (Fig. 9C, right). Among HY TCR-negative CD8 T cells in RASA1-deficient mice, CD44lo T cells are reduced in number specifically (Fig. 9D). Likewise, the reduced number of CD4 T cells in the periphery of RASA1-deficient HY TCR Tg mice can be explained by a specific reduction in the number of CD44lo T cells (Fig. 9D).
Functional responses of peripheral RASA1-deficient T cells

To determine whether RASA1 regulates TCR-induced activation of the Ras–MAPK pathway in peripheral T cells, naïve CD44^2 CD4^ peripheral T cells were purified from spleens and LNs of AND TCR Tg mice and stimulated with PCC peptide-pulsed I-E^k APC for different times in vitro. Activation of ERK-MAPK was then determined by phospho-flow cytometry and Western blotting (Fig. 10A, 10B). As shown, PCC peptide-pulsed APC-induced digital ERK activation responses in control and RASA1-deficient T cells that were similar in nature, both in terms of magnitude and duration. In accordance with these findings, amounts of the IL-2 cytokine synthesized by naïve AND TCR Tg T cells in response to PCC peptide-APC stimulation were the same between RASA1-deficient and control mice (Fig. 10C). Some reduction in the extent of PCC peptide–APC-induced proliferation of naïve AND RASA1-deficient T cells was observed (Fig. 10D); however, this difference in proliferation was only modest.

We also examined activation-induced cell death of RASA1-deficient naïve T cells. For this purpose, naïve AND T cells were stimulated with PCC peptide–APC and IL-2. After 2 d, cells were then replated in medium lacking IL-2, FBS, or both, and the extent of T cell death was determined after an additional 2 d culture (Fig. 10E). No difference in the extent of T cell death was observed between RASA1-deficient and control T cells. Likewise, no difference in T cell death was observed in similar assays in which the death of T cells was induced by restimulation with CD3 and CD28 Abs (data not shown).

In addition to examining AND TCR Tg T cells, we also examined ERK activation responses, cytokine synthesis, proliferation, and death of RASA1-deficient female HY TCR Tg T cells stimulated with HY peptide and H-2D^k APC and of non-TCR Tg T cells stimulated with CD3 and CD28 Abs. In each case, responses were similar to those observed with control T cells (data not shown). We conclude that RASA1 is dispensable as a regulator of each of these responses of peripheral T cells.

Homeostatic proliferation and survival of naive RASA1-deficient T cells

Based on findings in HY TCR Tg mice, we considered the possibility that a primary defect in the ability of naïve T cells to undergo homeostatic expansion accounts for the reduced number of these cells in the periphery of RASA1-deficient mice (29, 30). To examine this possibility directly, CFSE-labeled naïve CD8 T cells from non-TCR Tg RASA1-deficient or control wild type mice were injected into wild type recipients that had been rendered immunodeficient by sublethal irradiation. After 5 d, dilution of

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**Figure 7.** Effect of induced loss of RASA1 on peripheral T cell numbers. All experiments were performed with littermate rasa1^fl/fl and rasa1^fl/fl ub-ert2cre mice. A, Representative Western blot showing expression of RASA1 in spleens and purified splenic CD4^ and CD8^ T cells from mice injected with TM at 6 wk of age. Western blots were performed 8 wk after TM administration. B, Representative two-color flow cytometry plots (left) showing CD4 and CD8 staining on splenocytes of mice either not injected with TM (−TM) or injected with TM (+TM) at 6–8 wk of age. Analyses were performed at 3–4 mo of age. The bar graphs on the right show mean number plus 1 SEM of the indicated splenic T cell populations in mice without TM treatment (rasa1^fl/fl, n = 8; rasa1^fl/fl ub-ert2cre, n = 6) and with TM treatment (rasa1^fl/fl, n = 15; rasa1^fl/fl ub-ert2cre, n = 16). Statistical significance was determined using the Student two-sample t test. **p < 0.01.
donor T cell CFSE fluorescence was assessed to determine the extent of homeostatic proliferation (Fig. 11A). As shown, RASA1-deficient naive CD8 T cells were able to undergo homeostatic proliferation to the same extent as wild type naive CD8 T cells in this model. Furthermore, RASA1-deficient T cells proliferated to the same extent as wild type T cells in an alternative model of homeostatic proliferation in which T cells were adoptively transferred to RAG-deficient immunodeficient recipients (data not shown). Thus, a primary defect in homeostatic proliferation potential is unlikely to account for the paucity of naive T cells in RASA1-deficient mice.

An alternative explanation for the reduced numbers of naive T cells in RASA1-deficient mice is impaired survival in the periphery (29, 30). As a result, we asked whether naive T cells in RASA1-deficient mice showed increased levels of annexin V staining upon isolation from mice. The most clear cut results were obtained in the HY TCR Tg model (Fig. 11B). As shown, CD44low HY TCR-negative CD8 T cells from RASA1-deficient HY TCR Tg mice showed much higher amounts of annexin V staining compared with the same cell population in wild type HY TCR Tg mice. In contrast, memory CD44high HY TCR-negative CD8 T cells from RASA1-deficient HY TCR Tg mice showed the same amount of annexin V staining as their counterparts in wild type mice. This pattern of annexin V staining was also observed among CD4 T cells in HY TCR Tg mice. Thus, CD4 CD44low T cells showed increased annexin V staining in RASA1-deficient animals, whereas memory CD4 T cells did not (Fig. 11B). These findings strongly support the conclusion that the diminished number of CD44low T cells in RASA1-deficient mice is a consequence of decreased survival of these T cells in vivo.

Survival of naive T cells in the periphery is known to be mediated by the cytokine IL-7 after TCR recognition of self MHC peptide complexes (29, 30). Because the loss of RASA1 did not appear to affect TCR induced responses of naive peripheral T cells, we asked instead whether RASA1-deficient naive T cells showed impaired responsiveness to IL-7. For this purpose, whole T cells from RASA1-deficient and control non-TCR Tg mice were cultured in the presence or absence of IL-7. After 3 d, cell viability was then assessed (Fig. 11C). As shown, IL-7 promoted the survival of naive CD4 and CD8 T cells from both types of mice, but was clearly less efficient at promoting survival of CD44low RASA1-deficient T cells. In contrast, IL-7 promoted the survival of memory phenotype CD4 and CD8 T cells in control and RASA1-deficient mice to the same extent. We conclude, therefore, that impaired IL-7–mediated survival of naive T cells is the principal reason for the reduced numbers of naive T cells in RASA1-deficient mice.

Discussion

Knowledge of which RasGAPs regulate Ras activation in T cells is incomplete. To this end, we asked whether RASA1 functioned as regulator of Ras in T cells. Initial analysis of thymocyte differentiation in RASA1-deficient non-TCR Tg mice indicated that RASA1 might not play a role in positive selection. However, when thymocyte differentiation was examined in RASA1-deficient AND TCR Tg mice, a negative regulatory role for RASA1 in the positive
selection of CD4 SP T cells was revealed. Thus, despite total numbers of DP and mature CD4 SP cells being reduced in RASA1-deficient mice in this model, ratios of the number of postpositive selection to prepositive selection thymocytes were significantly increased. Furthermore, levels of CD69 expression were consistently increased upon preselection thymocytes in RASA1-deficient mice. It is unclear why a role for RASA1 in positive selection of CD4 T cells is apparent in AND TCR Tg mice but not for polyclonal CD4 T cells in non-TCR Tg mice. However, a relatively high affinity of the AND TCR for self peptide-MHC is likely a contributing factor. Only under such conditions might RASA1 contribute to regulation of the Ras-MAPK response during the course of positive selection.

Importantly, increased positive selection in the absence of RASA1 was associated with increased ERK signal transduction in prepositive and postpositive selection thymocytes. MAPK are known critical regulators of thymocyte positive selection (7). Expression of MAPK is essential in order for positive selection to occur (4). Furthermore, Tg mice that express a constitutively active mutant form of ERK2 known as sevenmaker in the T cell lineage show increased positive selection of CD4 SP cells but not CD8 SP T cells (31). Therefore, increased MAPK activation is likely the driving force for augmented positive selection of CD4 SP T cells in the absence of RASA1.

Regarding other major checkpoints in thymocyte differentiation, the loss of RASA1 did not appear to influence DN-to-DP transi-

**FIGURE 9.** Peripheral T cells in T cell-specific RASA1-deficient female HY TCR Tg mice. All analyses were performed on splenocytes or splenic T cells or thymocytes of littermate *rasa1*<sup>fl/fl</sup> and *rasa1<sup>fl/fl</sup> plck-cre* female HY TCR Tg mice. A, Representative Western blot showing expression of RASA1 in purified CD8 splenic T cells from female HY TCR Tg mice. B, Representative flow cytometric plots of CD4 and CD8 expression (left) and mean number plus 1 SEM of CD4 and CD8 T cells (right) in the spleens of female HY TCR Tg mice (*rasa1<sup>fl/fl</sup>, n = 8; *rasa1<sup>fl/fl</sup> plck-cre, n = 9). C, Representative histograms in the top panels show expression of the T3.70 marker on thymic CD8 SP T cells and splenic CD8 T cells of female HY TCR mice. Percentages of CD8 T cells that express the HY TCR (T3.70hi cells) are indicated. The bar graphs below show the mean number plus 1 SEM of the indicated thymic CD8 SP and splenic CD8 T cell populations (*rasa1<sup>fl/fl</sup>, n = 8; *rasa1<sup>fl/fl</sup> plck-cre, n = 9). D, Histograms show representative expression of CD44 upon the indicated splenic T cell populations of female HY TCR Tg mice. Filled and bold histograms represent *rasa1<sup>fl/fl</sup>* and *rasa1<sup>fl/fl</sup> plck-cre* mice respectively. The bar graph shows the mean number plus 1 SEM of the indicated populations in spleens (*rasa1<sup>fl/fl</sup>, n = 8; *rasa1<sup>fl/fl</sup> plck-cre, n = 9). Statistical significance was determined using the Student two-sample *t* test. **p < 0.01, ***p < 0.001.
tion in any of the examined models, despite RASA1 being well expressed in DN cells. DN-to-DP transition is also known to be critically dependent on activation of the Ras–MAPK pathway (3, 4). Therefore, it remains to be identified which RasGAP regulates Ras activation during DN-to-DP transition. In addition, no evidence was obtained that the loss of RASA1 increases negative selection or that RASA1 is required for negative selection of thymocytes. The lack of a role for RASA1 in negative selection is consistent with previous evidence that Ras–MAPK signaling pathway is not required for this event (6, 31–33).

In peripheral T cells, no evidence was obtained that RASA1 functions as a negative regulator of Ras activation in response to stimulation with agonist peptide–MHC complexes. Consequently, upon agonist peptide-MHC stimulation, RASA1-deficient T cells produced normal levels of cytokines, showed only a modest decrease in cellular proliferation, and showed appropriate levels of activation-induced cell death. However, numbers of naive CD4 and CD8 T cells were substantially reduced (by 50–90%) in the periphery of RASA1-deficient mice. Such a deficit could not be accounted for by impaired homeostatic proliferation, but ostensibly by an increased susceptibility of naive T cells to apoptotic cell death. Studies performed in vitro revealed that IL-7–mediated survival of naive T cells is impaired in the absence of RASA1. Survival of naive T cells in vivo is known to depend on IL-7 (29,
Therefore, this finding is likely to account for the diminished number of naive T cells in RASA1-deficient mice. Because gross alterations in thymic development were not apparent in non-TCR Tg mice, it is likely that the diminution of naive T cell number results from loss of RASA1 expression in that cell type. However, we cannot formally exclude the possibility that the reduced number of naive T cells in the periphery of RASA1-deficient mice is consequent to an altered T cell developmental program.

At the molecular level, how RASA1 promotes IL-7–mediated survival of naive T cells is unknown. IL-7 receptor signal transduction is known to involve activation of JAK protein tyrosine kinases leading to recruitment, phosphorylation, and activation of the STAT5 transcription factor (34). Activation of STAT5 proceeds normally in naive RASA1-deficient T cells (data not shown). Furthermore, in naive T cells from wild type mice, we have observed only minimal ERK activation in response to IL-7 stimu-
The authors have no financial conflicts of interest.

Disclosures

The authors have no financial conflicts of interest.

References


