The Journal of Immunology

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J Immunol 2005; 175:3133-3139; ;
doi: 10.4049/jimmunol.175.5.3133
http://www.jimmunol.org/content/175/5/3133

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Rap1-GTP Is a Negative Regulator of Th Cell Function and Promotes the Generation of CD4$^+$CD103$^+$ Regulatory T Cells In Vivo[1]

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The small GTPase Rap1 is transiently activated during TCR ligation and regulates integrin-mediated adhesion. To understand the in vivo functions of Rap1 in regulating T cell immune responses, we generated transgenic (Tg) mice, which express the active GTP-bound mutant Rap1E63 in their T lymphocytes. Although Rap1E63-Tg T cells exhibited increased LFA-1-mediated adhesion, ERK1/2 activation and proliferation of Rap1E63-Tg CD4$^+$ T cells were defective. Rap1E63-Tg T cells primed in vivo and restimulated with specific Ag in vitro, exhibited reduced proliferation and produced reduced levels of IL-2. Rap1E63-Tg mice had severely deficient T cell-dependent B cell responses, as determined by impaired Ig class switching. Rap1E63-Tg mice had an increased fraction of CD4$^+$CD103$^+$ regulatory T cells (Treg), which exhibited enhanced suppressive efficiency as compared with CD4$^+$CD103$^+$ Treg from normal littermate control mice. Depletion of CD103$^+$ Treg significantly restored the impaired responses of Rap1E63-Tg CD4$^+$ T cells. Thus Rap1-GTP is a negative regulator of Th cell responses and one mechanism responsible for this effect involves the increase of CD103$^+$ Treg cell fraction. Our results show that Rap1-GTP promotes the generation of CD103$^+$ Treg and may have significant implications in the development of strategies for in vitro generation of Treg for the purpose of novel immunotherapeutic approaches geared toward tolerance induction. *The Journal of Immunology,* **2005, 175: 3133–3139.**

A member of the Ras superfamily, Rap1 was discovered as a gene product that reverted K-Ras-induced transformation (1). Because Ras and Rap1 share an identical effector domain, it was hypothesized that Rap1 reverts Ras-induced transformation by competing for common downstream effectors (2–4). However, it is today known that Rap1 functions independently of Ras and initiates active signaling events: in platelets, Rap1 augments fibrinogen binding to $\alpha_{II}\beta_3$ integrin (5, 6); and in neutrophils, it is involved in superoxide generation and initiation of the respiratory burst (7). In B lymphocytes Rap1 is activated by BCR and in fibroblasts by epidermal and platelet-derived growth factor receptor ligation (8, 9). Rap1 is involved in integrin-mediated adhesion in various cell types (10–14).

In T lymphocytes, transient activation of Rap1 and accumulation of Rap1-GTP at the T cell:APC interface is one of the physiologic consequences of TCR ligation and regulates LFA-1:ICAM-1 mediated signals (10, 15). TCR-, CD98-, and CD31-induced adhesion requires Rap1 activation. TCR- and CD98-induced adhesion is blocked by dominant negative Rap1N17, and CD31-induced adhesion is inhibited by Rap1N17 and Rap1 GTPase-activating protein (Rap1GAP) that inactivates Rap1 (10, 11, 16). In contrast to these effects of Rap1 that are anticipated to regulate T cell activation in a positive manner, constitutive expression of Rap1-GTP in various T cell systems inhibits IL-2 transcription (15, 17, 18) and results in augmentation of the cell cycle inhibitor p27^{kip1} (15). Increase of active Rap1 is also detected in anergic cells that are incapable of responding to antigenic stimulation (17). These observations suggest that Rap1 is involved in T cell responses but its role in the regulation of immune responses of the intact host remains unclear.

We generated transgenic (Tg)[3] mice that express the active Rap1 mutant Rap1E63 in T cells. In this study we show that Rap1E63-Tg thymocytes were capable of activating Ras and ERK1/2 and exhibited enhanced LFA-1:ICAM-1-mediated adhesion and increased proliferation. Surprisingly, although peripheral Rap1E63-Tg CD4$^+$ T cells also exhibited enhanced LFA-1:ICAM-1-mediated adhesion, they had impaired activation of ERK1/2 and defective Th cell responses in vivo. Rap1E63-Tg mice had a striking increase of the CD4$^+$CD103$^+$ regulatory T cell (Treg) fraction. Rap1E63-Tg CD4$^+$CD103$^+$ Treg had enhanced suppressive efficiency as compared with CD4$^+$CD103$^+$ Treg from normal littermate control (NLC) mice. Depletion of CD4$^+$CD103$^+$ Treg significantly restored the impaired ERK1/2 activation, proliferation and IL-2 production of Rap1E63-Tg CD4$^+$ T cells. Our results show that Rap1-GTP is a negative regulator of Th cell responses and promotes the generation of CD103$^+$ Treg in vivo.

Materials and Methods

Generation of Rap1-GTP (E63) Tg mice

A 325-bp cDNA fragment of human sequence of constitutively active Rap1A (Rap1E63 mutant, kindly provided by Dr. H. Kitayama, Kyoto

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Received for publication March 4, 2005. Accepted for publication June 10, 2005.

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[1] This work is supported by National Institutes of Health Grants AI 43552 and AI 46548.

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[3] Abbreviations used in this paper: Tg, transgenic; Treg, T regulatory cell; WT, wild type; SP, single positive; DP, double positive; TNP, 2,4,6-trinitrophenol.

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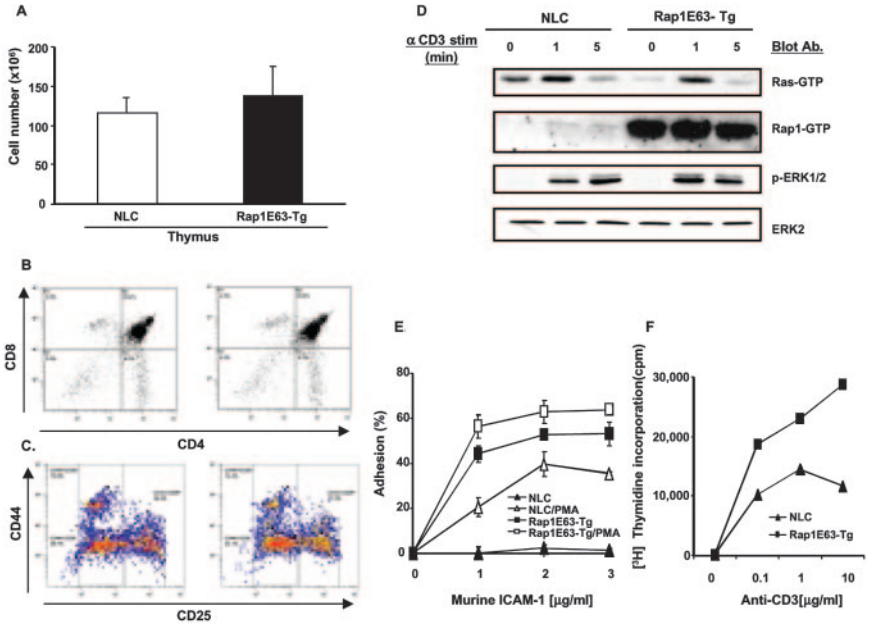

FIGURE 1. Rap1E63-Tg thymocytes activate Ras-ERK1/2 and exhibit enhanced LFA-1:ICAM-1 mediated adhesion and CD3-mediated proliferation. *A*, Absolute thymocyte numbers were examined using thymi from age-matched (6- to 12-wk-old) Rap1E63-Tg and NLC mice. Results are mean values obtained from 10 independently analyzed mice in each group. *B*, T cell differentiation was assessed by staining thymocytes with CD4- and CD8-specific mAbs and analyzed by flow cytometry. The percentage of T cell subsets is shown in dot plot quadrants. Results are representative of 10 independently analyzed mice in each group. *C*, Thymocytes were stained with anti-CD4, anti-CD8, anti-CD25, and anti-CD44 Abs. The expression of CD25 and CD44 were analyzed on gated $CD4^-CD8^-$ population. *D*, Thymocytes were stimulated by CD3 cross-linking. Activation of Ras and Rap1 was examined by pull-down assay. Activation of ERK1/2 was determined by immunoblot with phospho-specific Ab, followed by immunoblot with ERK2 Ab to confirm equal protein loading. *E*, Thymocytes (5×10^5 cells/well) were untreated or treated with PMA and adhesion on ICAM-1 was examined. Adhesion is shown as a percentage calculated using ((adhesive thymocytes background)/(total cell input)). Data show mean value and SD of triplicates and represent two independent experiments. *F*, Thymocytes (1×10^6 cells/well) were cultured with anti-CD3 mAb (1 μg/ml) for 4 days and proliferation was assessed. Results show mean value and SD of triplicates and are representative of three independent experiments.

University, Kyoto, Japan) was subcloned into the *Sma*I site of a Tg expression vector that contained the promoter and the locus control region of the gene encoding human CD2. This approach results in selective expression of the transgene in T cells (19). The resulting plasmid was cut with the restriction enzymes *Kpn*I and *Not*I to generate a 10.1-kb fragment that contained the CD2 promoter, intron sequences, locus control region, and Rap1E63 cDNA. This fragment was used to generate Tg mouse lines using standard protocols. Mice carrying the transgene were identified by Southern blot of genomic DNA. Stable Rap1E63 lines were established by backcrossing transgene-carrying founder mice with C57BL/6 mice (Charles River Breeding Laboratories). Mice were bred and maintained in the Dana-Farber Cancer Institute animal housing facility (Boston, MA).

Cell isolation

Lymphocytes were prepared from pooled peripheral lymph node and spleen. $CD4^+$ cells were prepared by positive selection with Macs beads (Miltenyi Biotec). The $CD103^+$ T cell subpopulation was separated from purified $CD4^+$ T cells by staining with FITC-labeled anti-CD103 followed by fluorescence-activated cell sorting as indicated in each experiment.

Flow cytometric analysis

Surface Ag expression was determined with Abs specific for CD8a, CD4, CD45RB, CD62L, CD69, CD44, CD54, LFA-1 (BD Pharmingen), CD25, and CD103 (eBioscience) and analyzed by flow cytometry.

Proliferation of $CD4^+$ T cells and Treg-mediated suppression

For proliferation, purified $CD4^+$ T cells (1×10^6 cells/ml, 50 μl/well) from Rap1E63-Tg or NLC mice and T cell-depleted, mitomycin C-treated APCs (10×10^6 cells/ml, 50 μl/well) were cultured for 1–5 days in the presence of 1 μg/ml anti-CD3 mAb (2C11). [^{3}H]Thymidine incorporation was assessed during the last 8 h of culture. For assessment of suppressive activity of $CD103^+CD4^+$ T cells, $CD4^+CD25^-CD103^-$ wild-type (WT) syngeneic T cells (5×10^4 cells/well) were cultured for 96 h in flat-bottom 96-well plates containing 5×10^5 T cell-depleted APCs, 1 μg/ml anti-CD3 mAb, and various numbers of sorted $CD4^+CD103^+$ T cells from NLC or Rap1E63-Tg mice. Proliferation of responder T cells was determined by [^{3}H]thymidine incorporation for the last 8 h of culture.

Cytokine analysis

To measure IL-2 production, cell cultures were set up as in proliferation assay. Supernatants were harvested and assessed by ELISA using anti-IL-2 Ab according to the manufacturer's instructions (BD Pharmingen).

In vivo T cell priming and in vitro restimulation

Mice were immunized with 2,4,6-trinitrophenol (TNP)-OVA in CFA in the footpad. Lymphocytes from the draining lymph nodes were harvested 10 days later and were either stimulated with OVA or $CD4^+$ T cells were purified and were stimulated in vitro with OVA in the presence of mitomycin C-treated APCs from WT unimmunized mice. Supernatants were analyzed for IL-2 production by ELISA.

Ab responses and basal Ig detection

For T cell-dependent B cell responses, mice were immunized with 100 μg of TNP-OVA (Biosearch Technologies) in IFA (Sigma-Aldrich) in the footpad, were bled 7 and 14 days later and the sera were tested for isotype-specific anti-TNP Abs by ELISA. Basal Ig levels were determined by ELISA using isotype-specific goat Abs to mouse Ig (BD Pharmingen). Results were expressed as OD_{450} absorbance values. For T cell-independent B cell responses, mice were immunized with 100 μg of TNP-Ficoll in IFA in the footpad. Mice were bled on days 7 and 14 after immunization and the sera were tested for isotype-specific anti-TNP Abs by ELISA. The results of 7-day Ig levels are shown.

Real-time quantitative PCR analysis

Total RNA was extracted from 2 to 6×10^6 subsets of $CD4^+$ T cells using RNA-Bee (Tel-Test). Reverse transcription was done using superscript II RT kit (Invitrogen Life Technologies). Foxp3 mRNA levels were quantified by real-time PCR, using primers and an internal fluorescent TaqMan probe specific for Foxp3 or GAPDH (Applied Biosystems).

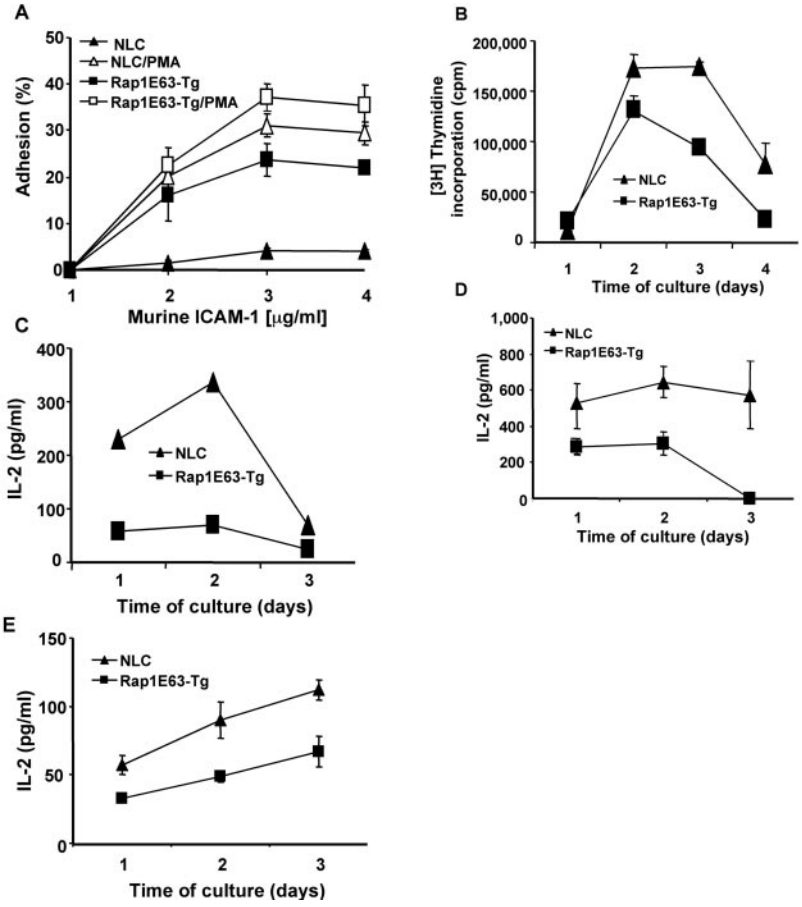

FIGURE 2. Rap1E63-Tg CD4$^+$ T cells exhibit enhanced LFA-1:ICAM-1 mediated adhesion but defective primary and secondary responses. *A*, Purified peripheral CD4$^+$ T cells (2.5×10^5 cells/ml) from Rap1E63-Tg and NLC mice were untreated or treated with PMA, and adhesion on ICAM-1 was examined as described in *Materials and Methods*. Results show mean value and SD of triplicates and represent three independent experiments. *B* and *C*, CD4$^+$ T cells (5×10^4 cells/well) were stimulated with anti-CD3 mAb (1 μg/ml) and APCs (5×10^5 cells/well) from WT syngeneic mice for the indicated time intervals, and proliferation and IL-2 production were assessed. The same pattern of response was observed with a wide range of anti-CD3 mAb concentrations (data not shown). Results show mean value and SD of triplicates and represent five independent experiments. *D*, Mice were immunized with TNP-OVA, lymphocytes were harvested from the draining lymph nodes and stimulated with OVA. Supernatants were analyzed for IL-2 production. *E*, CD4$^+$ T cells were purified from TNP-OVA immunized mice and were stimulated with OVA and mitomycin C treated APCs from unimmunized WT syngeneic mice. Supernatants were analyzed for IL-2 production. Results show mean value and SD of duplicates and represent three independent experiments.

Immunoblotting and pull-down assay

Preparation of cell lysates, SDS-PAGE, and immunoblot were done as described before (17). Phospho-ERK1/2 Ab was from Santa Cruz Biotechnology, ERK1/2 and ERK2 Abs were from BD Transduction Laboratories. Rap1GTP and RasGTP were detected by pull-down with GST-RalGDS-RBD and GST-Raf1-RBD, respectively (9). Rap1 Ab was from Santa Cruz Biotechnology and Ras Ab was from Oncogene Research Products.

Analysis of ERK activation by flow cytometry

The indicated T cell populations were either left unstimulated or were stimulated by cross-linking of CD3 and CD28; subsequently the cells were fixed in 1% formaldehyde solution and permeabilized by 90% ice-cold methanol on ice for 30 min. The cells were washed with 0.5% BSA-PBS, stained with Alexa Fluor 488-conjugated phospho-ERK (Cell Signaling Technology) according to the manufacturer's instructions, and analyzed by flow cytometry.

Cell adhesion

Assessment of cell adhesion was done as previously described (19). Briefly, flat-bottom Maxisorp 96-well plates (Nunc) were uncoated (as control) or precoated with ICAM-1/Fc (R&D Systems) overnight at 4°C. The plates were washed with PBS and nonspecific binding sites were blocked with 2% denatured BSA for 1 h at 37°C. Freshly isolated thymocytes or purified peripheral CD4$^+$ T cells were labeled with 1 μM BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; Calbiochem) for 30 min at 37°C, followed by further washes with cold 10% FCS-RPMI 1640. The cells were added to precoated plates and incubated for 1 h at 37°C. When indicated, PMA (250 ng/ml) was added during incubation. Adhesion was quantified using Spectra MAX (Molecular Devices). Specific adhesion was obtained by subtracting background adhesion (mean reading for wells coated with PBS only) from the reading for each well. This specific adhesion was expressed as a percentage of the total emission before the incubation, that is, the percentage total cell input added to the well.

Results

Increased adhesion and proliferation in Rap1E63-Tg thymocytes

Rap1E63-Tg and NLC mice had no statistically significant difference in absolute thymocyte numbers (Fig. 1*A*). Differentiation profiles of double positive (DP) and single positive (SP) thymocytes and expression of CD44 (detected in double negative stage I and stage II) were comparable between Rap1E63-Tg and NLC mice (Fig. 1, *B* and *C*). Expression profiles of CD5 and CD69 in the DP and SP populations were also comparable between Rap1E63-Tg and NLC mice (data not shown).

It is controversial whether active Rap1 inhibits Ras and ERK activation (2, 9, 19). We examined activation of Ras and ERK in Rap1E63-Tg thymocytes. Despite the increased constitutive expression of Rap1-GTP, thymocytes of Rap1E63-Tg mice were capable of activating Ras and ERK1/2 at levels comparable to NLC thymocytes (Fig. 1*D*). Because Rap1-GTP is known to induce LFA-1-mediated adhesion (10, 11, 16), we examined the ability of Rap1E63-Tg thymocytes to adhere on recombinant ICAM-1. Adhesion on ICAM-1 was dramatically increased in Rap1E63-Tg thymocytes and was slightly enhanced by PMA (Fig. 1*E*). In addition, proliferation in response to anti-CD3 stimulation was enhanced in Rap1E63-Tg thymocytes (Fig. 1*F*). Thus, in thymocytes Rap1-GTP enhances LFA-1-mediated adhesion and cellular proliferation, consistent with previous report (19).

Defective primary responses of peripheral CD4$^+$ T cells in Rap1E63-Tg mice

Active Rap1 has a critical role in regulating T cell responses and anergy (15, 17, 18), a phenomenon well established for mature CD4$^+$

T cells. For this reason, we examined the effects of Rap1E63 on the responses of peripheral $CD4^+$ T cells. Similarly to Rap1E63-Tg thymocytes, Rap1E63-Tg $CD4^+$ T cells exhibited increased adhesion on ICAM-1 (Fig. 2*A*). Surprisingly, Rap1E63-Tg $CD4^+$ T cells had reduced anti-CD3-mediated proliferation ($p = 0.0008$) and IL-2 production as compared with NLC $CD4^+$ T cells (Fig. 2, *B* and *C*). The diminished responses were not due to increased activation-induced apoptosis in Rap1E63-Tg T cells (data not shown). These results indicate that in mature peripheral $CD4^+$ T cells, Rap1-GTP enhances LFA-1-mediated adhesion, but despite this, inhibits proliferation and IL-2 production.

Impaired in vivo Th cell responses in Rap1E63-Tg mice

To assess responses of Rap1E63-Tg $CD4^+$ T cells in vivo, we examined Ag-specific recall responses. We immunized Rap1E63-Tg and NLC mice with a T cell-dependent Ag, TNP-OVA. Subsequently, T cells from immunized mice were restimulated in vitro with OVA and IL-2 production was examined. IL-2 production by restimulated Rap1E63-Tg T cells was reduced (Fig. 2*D*) suggesting that Rap1E63 impairs T cell activation in vivo. To determine definitively that impaired Ag-specific recall responses were due to defective in vivo activation of $CD4^+$ T cells, we purified $CD4^+$ T cells from immunized mice and stimulated them with OVA and mitomycin C-treated APCs. IL-2 production of $CD4^+$ T cells from immunized Rap1E63-Tg mice was impaired ($p = 0.005$) (Fig. 2*E*).

$CD4^+$ Th cells provide help for B cell production of all subsets of Abs and for Ig class switch. To further evaluate in vivo helper function of Rap1E63-Tg $CD4^+$ T cells we examined T cell-dependent B cell responses by analyzing humoral immune responses (20). We immunized Rap1E63-Tg and NLC mice with TNP-OVA and measured anti-TNP Ig isotypes. Rap1E63-Tg and NLC mice had comparable basal levels of IgM, IgG1, and IgG2a (Fig. 3*A*). Following immunization, production of anti-TNP IgM was reduced by 2-fold ($p = 0.05$), and production of anti-TNP IgG was reduced by 3/4-fold ($p = 0.009$) in Rap1E63-Tg mice compared with NLC (Fig. 3*B*). Production of anti-TNP IgG2a was reduced by 2/3-fold ($p = 0.04$), and IgG1 was reduced by 5-fold ($p = 0.005$) and was almost undetectable in Rap1E63-Tg mice (Fig. 3*B*). These results show that after immunization, Ab production and more strikingly IgG1 and IgG2a isotype class switching was deficient in Rap1E63-Tg mice, further supporting the conclusion that Rap1E63-Tg $CD4^+$ T cells have impaired helper function.

If Rap1E63 altered the capacity of T cells to provide efficient T cell help, then B cell responses to T cell-independent Ags should remain unaltered in the Rap1E63-Tg mice. To address this issue we challenged Rap1E63-Tg and NLC mice with trinitrophenol conjugated to Ficoll (TNP-Ficoll), a classical T cell independent Ag (21). Production of anti-TNP IgM and anti-TNP IgG occurred to the same extend in Rap1E63-Tg and NLC mice (Fig. 3*C*). Thus, B cells are intrinsically normal in Rap1E63-Tg mice and the observed defects in isotype switching are probably due to alterations in Th cell activity.

Increase of $CD4^+CD103^+$ Treg fraction in Rap1E63-Tg mice

We determined previously by suppression subtractive hybridization that Rap1-GTP regulates gene expression. CD103 was one of the genes up-regulated by Rap1-GTP (22). We examined whether CD103 expression was altered in $CD4^+$ T cells of Rap1E63-Tg mice. We detected increase in mRNA expression (data not shown) and a 4-fold increase in the $CD4^+CD103^+$ T cell fraction in spleens and lymph nodes of Rap1E63-Tg mice ($p = 0.007$) (Fig. 4*A*). There was also a lower but consistent increase in the $CD8^+CD103^+$ T cell fraction in spleens and lymph nodes of Rap1E63-Tg mice (data not shown). Because the present report is focused on the role of Rap1-GTP on Th cell function, further studies on these $CD8^+$ cells will not be included in this study.

CD103 defines a subset of peripherally generated Treg with potent suppressive function (23–25). Therefore, we examined whether $CD4^+CD103^+$ T cells in Rap1E63-Tg mice had the molecular and functional properties of Treg. Immunophenotype analysis showed expression of $CD45RB^{low}$, $CD44^{high}$, $CD54^{high}$, $CD62L^{low}$, and LFA-1^{high} (data not shown), which were findings

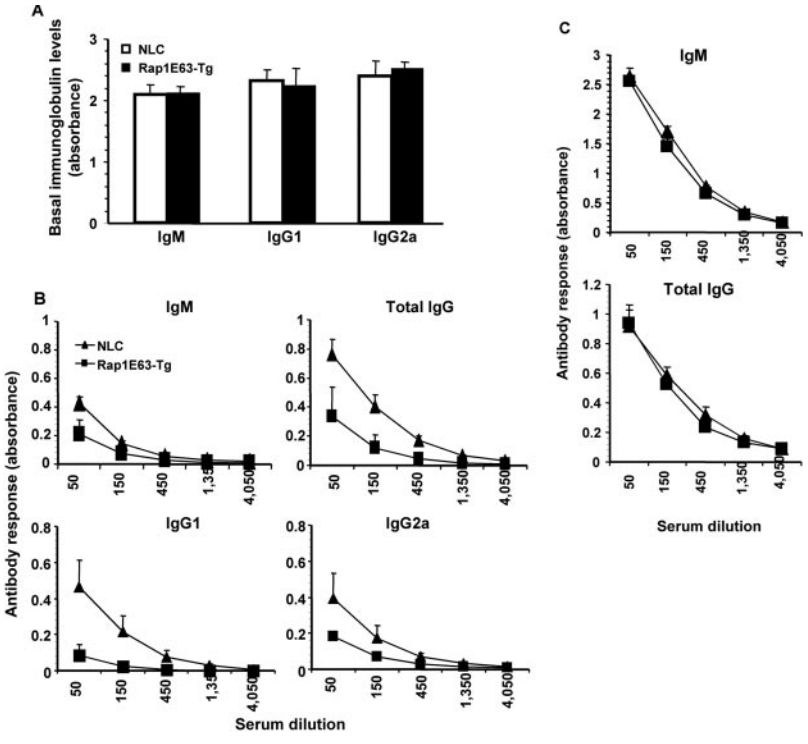

FIGURE 3. Rap1E63-Tg mice have impaired Ab production and isotype class switching in response to T cell-dependent Ag. *A*, Rap1E63-Tg and NLC mice have comparable basal Ig levels. *B*, Mice were immunized with 100 μg of TNP-OVA in IFA and 7 days later the sera were tested for isotype-specific anti-TNP Abs by ELISA. Results show mean value and SD of three individual mice per group. *C*, Mice were immunized with 100 μg of TNP-Ficoll in IFA, and 7 days later the sera were tested for TNP-specific IgM and IgG by ELISA. The results show mean value and SD of three individual mice per group.

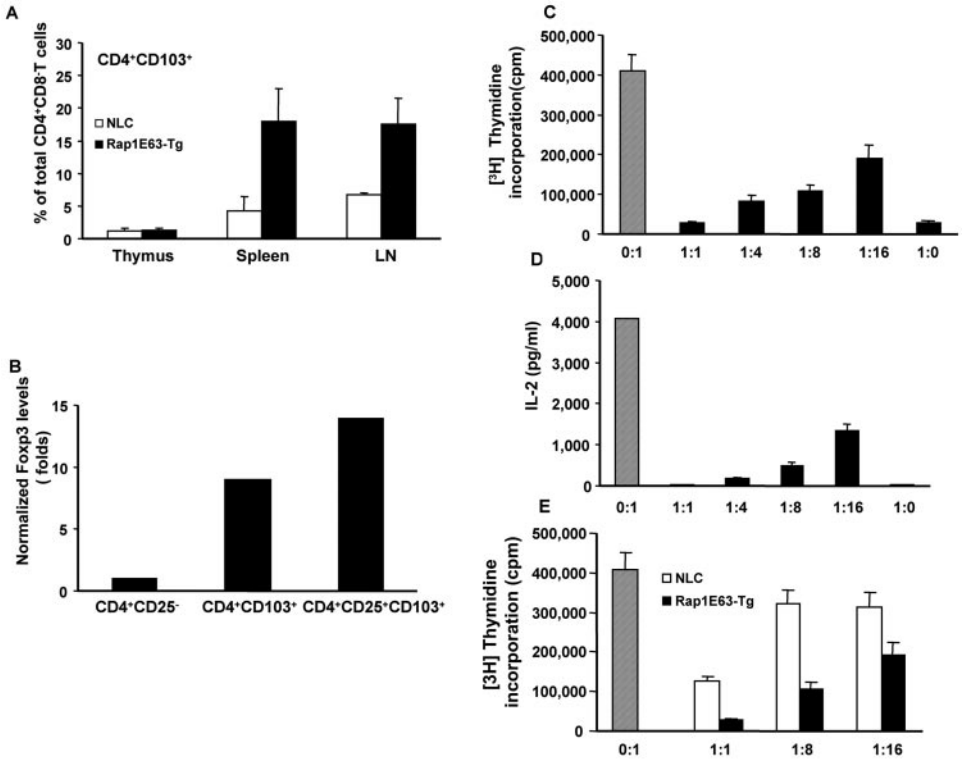

FIGURE 4. Increased percentage of $CD4^+CD103^+$ T cells, CD8 with properties of Treg are present in spleens and lymph nodes of Rap1E63-Tg mice. *A*, Cells from thymi, spleens and lymph nodes (LN) were stained with CD4 and CD103-specific mAbs. Percentages of the $CD4^+CD103^+$ subset in total $CD4^+CD8^-$ T cells in each lymphoid organ are shown. Six individual Rap1E63-Tg mice were compared with six individual age-matched NLC mice. *B*, cDNA from the indicated T cell subsets was subjected to real-time quantitative PCR analysis to quantify expression of Foxp3 mRNA. *C*, Purified $CD4^+CD25^-$ T cells (5×10^4 cells/well) from WT mice were cocultured with anti-CD3 mAb (1 μg/ml), mitomycin C treated APCs (5×10^5 cells/well), and various numbers of sorted $CD4^+CD103^+$ Rap1E63-Tg T cells. Ratio 0:1 represents no addition of $CD4^+CD103^+$ Rap1E63-Tg cells (▨) and ratio 1:0 represents no addition of $CD4^+CD25^-$ T cells. Proliferation was assessed at 4 days and production of IL-2 was assessed in the supernatants at 3 days (*D*) of culture. *E*, Purified $CD4^+CD25^-$ T cells from WT mice were cocultured with anti-CD3 mAb, APCs (as in *C*), and various numbers of sorted $CD4^+CD103^+$ T cells from either Rap1E63-Tg (■) or NLC (□) mice. Ratio 0:1 represents no addition of $CD4^+CD103^+$ T cells (▨). Proliferation was assessed at 4 days.

consistent with the previously reported pattern of surface Ag expression in $CD4^+CD103^+$ Treg (24). $CD103^+$ cells within the entire $CD4^+$ T cell population and within the $CD4^+CD25^+$ subset expressed high levels of Foxp3 (Fig. 4*B*), which is the hallmark of Treg (26). $CD4^+CD103^+$ Rap1E63-Tg T cells were also potent inhibitors of proliferation and IL-2 production of $CD4^+CD25^-$ T cells (Fig. 4, *C* and *D*). Thus, $CD4^+CD103^+$ Rap1E63-Tg T cells have molecular and functional properties of Treg. Impressively, when compared with $CD4^+CD103^+$ cells from NLC mice, Rap1E63-Tg $CD4^+CD103^+$ T cells appeared to be more potent inhibitors of $CD4^+CD25^-$ T cells (Fig. 4*E*).

$CD4^+CD103^+$ Treg inhibit ERK1/2 activation and contribute to the decreased responses of peripheral $CD4^+$ T cells of Rap1E63-Tg mice

Our results showed that Rap1E63-Tg thymocytes exhibited increased proliferation compared with NLC thymocytes, whereas peripheral Rap1E63-Tg $CD4^+$ T cells exhibited reduced proliferation compared with NLC $CD4^+$ T cells. In addition, our data showed that Rap1E63-Tg thymocytes were capable of activating ERK1/2 to levels comparable to NLC thymocytes (Fig. 1*D*, *third panel*). Because of the distinct proliferative responses of Rap1E63-Tg thymocytes and peripheral Rap1E63-Tg $CD4^+$ T cells, we examined ERK1/2 activation in peripheral Rap1E63-Tg $CD4^+$ T cells. We used flow cytometry that allows quantification of ERK1/2 phosphorylation at a single cell level. Using peripheral $CD4^+$ T cells from Rap1E63-Tg and NLC mice, we observed that induction of ERK1/2 activation after anti-$CD3^+$ and anti-CD28 stimulation was impaired in Rap1E63-Tg $CD4^+$ T cells (Fig. 5*A*, *upper* and *middle row*). Because Rap1E63-Tg $CD4^+CD103^+$ T cells were potent inhibitors of $CD4^+CD25^-$ T cells (Fig. 4, *C* and *D*), we examined whether they might have a role in the impaired capacity of Rap1E63-Tg $CD4^+$ T cells to activate ERK1/2. To address this question, we examined responses of Rap1E63-Tg $CD4^+$ T cells after depletion of $CD4^+CD103^+$ T cells. As shown in Fig. 5*A* (*bottom row*), when $CD103^+$ Treg were depleted from Rap1E63-Tg $CD4^+$ T cells, ERK1/2 activation was significantly restored. Similar results were obtained when activation of ERK1/2 was examined by Western blot in Rap1E63-Tg and NLC $CD4^+$ T cells (Fig. 5*B*, *top panels*) and when the $CD103^+$ T cell subset was depleted from Rap1E63-Tg $CD4^+$ T cells (Fig. 5*B*, *bottom panels*). Consistent with the enhanced activation of ERK1/2, depletion of $CD103^+$ Treg cells significantly restored proliferation and IL-2 production in Rap1E63-Tg $CD4^+$ T cells (Fig. 5, *C* and *D*).

Our data showed that Rap1E63-Tg $CD4^+CD103^+$ Treg were more potent inhibitors than NLC $CD4^+CD103^+$ Treg (Fig. 4*E*). Therefore, one mechanism possibly responsible for the reduced activation and the impaired responses of Rap1E63-Tg $CD4^+$ T cells might be the presence of highly efficient Rap1E63-Tg Treg. To investigate this hypothesis, we depleted Treg and we compared responses of $CD4^+CD25^-CD103^-$ Rap1E63-Tg and NLC T cells. Impressively, even after depletion of Treg, proliferative responses

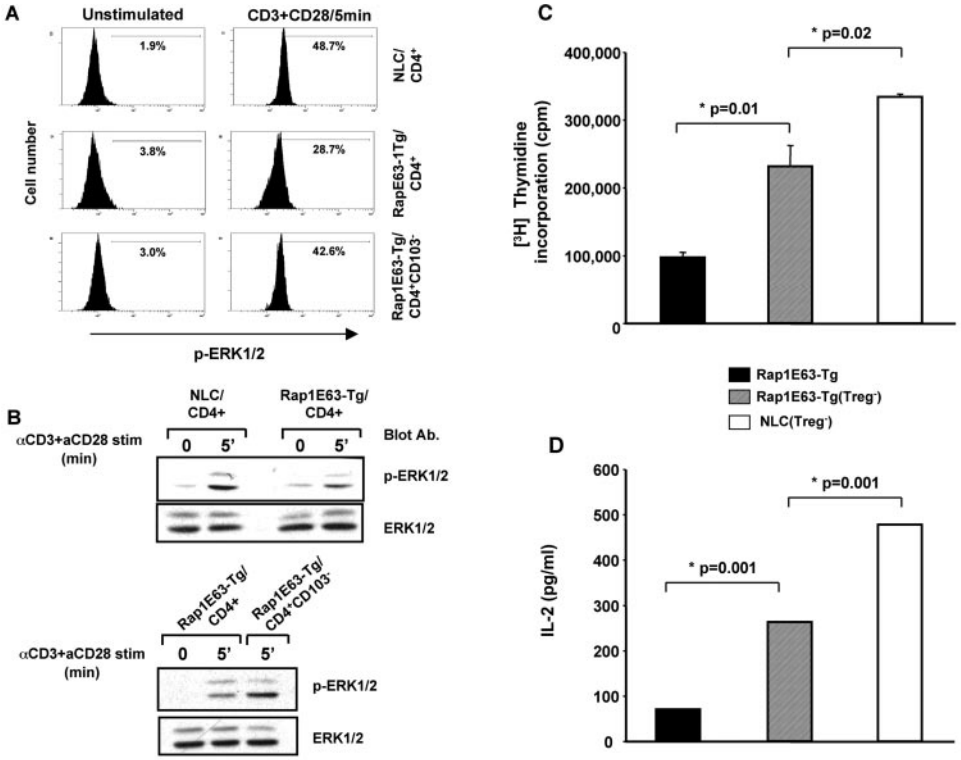

FIGURE 5. $CD4^+CD103^+$ Treg cells inhibit ERK1/2 activation, proliferation, and IL-2 production of Rap1E63-Tg $CD4^+$ T cells. *A*, The indicated T cell populations were either left unstimulated or were stimulated by CD3 and CD28 cross-linking, stained with Alexa Fluor 488-conjugated phospho-ERK, and analyzed by flow cytometry. Results are expressed as a percentage of phospho-ERK positive cells before (*left panels*) and after (*right panels*) 5 min of stimulation. *B*, The indicated T cell populations were stimulated by CD3 and CD28 cross-linking for 5 min and equal amounts of protein (50 μg) were analyzed by SDS-PAGE. Activation of ERK1/2 was determined by immunoblot with phospho-specific Ab, followed by immunoblot with ERK1/2 Ab to confirm equal protein loading. *C* and *D*, Rap1E63-Tg $CD4^+$ T cells (■), Treg-depleted Rap1E63-Tg $CD4^+$ T cells (▨) and Treg-depleted $CD4^+$ NLC cells (□) were cultured with anti-CD3 mAb (1 μg/ml) and WT syngeneic APC (5×10^5 cells/well). Proliferation was assessed at 3 days and IL-2 concentration was assessed in the supernatants at 2 days of culture. Results show mean value and SD of triplicates and represent three independent experiments.

and IL-2 production of Rap1E63-Tg $CD4^+$ T cells were diminished (Fig. 5, *C* and *D*), suggesting that an additional intrinsic defect was mediated by Rap1-GTP.

Discussion

Our present data provide evidence that Rap1 is a negative regulator of Th cell function in the intact host. First, Rap1E63-Tg $CD4^+$ T cells exhibited defects in proliferation and IL-2 production after in vitro stimulation. Second, Rap1E63-Tg $CD4^+$ T cells primed by immunization with a T cell-dependent Ag in vivo and restimulated with specific Ag in vitro, produced reduced levels of IL-2. Third, Rap1E63-Tg mice had impaired T cell-B cell collaboration in vivo as determined by defective Ab production and Ig class switching.

An additionally striking finding was that the Rap1-GTP transgene induced an increase of the $CD4^+CD103^+$ Treg fraction. $CD4^+CD103^+$ Treg cells are considered adaptive, peripherally generated Treg and are very potent suppressors of Ag-induced arthritis and wasting inflammatory bowel disease (23–25). Although the majority are $CD25^+$, $CD4^+CD103^+CD25^-$ Treg have been identified (23, 25). $CD4^+CD103^+$ Treg cells produce IL-10 and TGF-β but mediate their suppressive function by a contact-dependent mechanism. Our studies showed that Rap1E63-Tg $CD4^+CD103^+$ T cells have properties of $CD103^+$ Treg as they express high levels of Foxp3, inhibit responses of $CD4^+CD25^-$ T cells, and produce IL-10 and TGF-β when stimulated by CD3 plus CD28 ligation (our unpublished observation). Thus, Rap1-GTP may be involved in the generation of $CD103^+$ Treg in vivo.

$CD4^+CD103^+$ Treg does not seem to account exclusively for the impaired responses of Rap1E63-Tg $CD4^+$ T cells because after depletion of Treg, the remaining $CD4^+$ cells had a less prominent but still detectable defect in proliferation and IL-2 production. Several explanations of the defective responses can be entertained. First, it has been shown that the balance between LFA-1 and CD28-mediated signals determines the fate of T cells during antigenic stimulation. LFA-1 does not deliver a true costimulatory signal and cells that encounter TCR stimulation predominantly in the context of a LFA-1 become anergic (27). Second, recent reports suggest that the strength of TCR signal determines the recruitment of negative regulators at the immunological synapse one of which is CTLA-4 (28). Thus, increased strength of TCR signal in Rap1E63-Tg $CD4^+$ T cells due to augmented LFA-1-mediated adhesion may recruit CTLA-4 and/or other negative regulators in close proximity to the TCR, rendering T cells incapable of mounting competent immune responses (29). Regardless of the mechanism, our present data provide evidence that Rap1-GTP functions as a negative regulator of $CD4^+$ Th cell responses.

Interestingly, Rap1-GTP has a different role in regulating responses of thymocytes vs peripheral $CD4^+$ T cells. Rap1E63-Tg thymocytes had enhanced proliferation whereas peripheral Rap1E63-Tg $CD4^+$ T cells had reduced proliferation and IL-2 production, compared with the relevant NLC populations. One apparent explanation is that this difference may be due to the $CD4^+CD103^+$ Treg fraction that is increased within the peripheral $CD4^+$ T cells but not within the thymocytes of Rap1E63-Tg mice

(Fig. 4*A*). However, Rap1 may have distinct roles in thymocytes vs peripheral T cells, because it is differently regulated in these two cell populations: CD28 enhances CD3-dependent activation of Rap1 in thymocytes, but not in peripheral T cells; CD3-mediated Rap1 activation in thymocytes is Ca^{2+}-independent, whereas in peripheral T cells it is Ca^{2+}-dependent (30).

A previous study used a different active Rap1 mutant Rap1V12, to generate a T cell specific Rap1V12-Tg mice (19). Similar to our Rap1E63-Tg mice, Rap1V12-Tg mice had comparable thymic cellularity with NLC mice and their thymocytes exhibited increased integrin-mediated adhesion and increased proliferation. However, Rap1E63-Tg and RapV12-Tg mice had significant differences in the responses of peripheral T cells: Rap1E63-Tg $CD4^+$ T cells had impaired proliferation and IL-2 production, whereas proliferation of Rap1V12-Tg splenocytes was indistinguishable from that of NLC cells. Although the precise reasons of these differences cannot be determined, the two Rap1-GTP-Tg mouse lines were generated using different Rap1 mutants. This is an important issue, because Rap1E63 has five times stronger biological activity than Rap1V12 in mediating Rap1-GTP effects (31). Consistently, Rap1E63 is not subject to the effects of Rap1GAP and remains constitutively in the GTP-bound active state, whereas Rap1V12 is susceptible to the effects of several Rap1GAPs that convert this mutant into the inactive GDP-bound form (32).

A recent report further supports our findings on the role of Rap1-GTP as a negative regulator of T cell activation (33). Mice deficient for the signal-induced proliferation-associated gene-1 SPA-1, a Rap1GAP that inactivates Rap1, displayed increased Rap1 activation and impaired T cell responses. Similarly to Rap1E63-Tg mice, in SPA-1-deficient mice T cells primed with Ag in vivo and restimulated in vitro exhibited impaired recall responses. Defective T cell responses were also evident by impaired T cell-B cell collaboration in vivo that resulted in defective Ig class switching, similarly to our observations in Rap1E63-Tg mice. Taken together these studies provide compelling evidence that Rap1-GTP is a negative regulator of $CD4^+$ Th cell function.

Acknowledgments

We are grateful to John Daley and Susan Lazo for technical support with cell sorting.

Disclosures

The authors have no financial conflict of interest.

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