The Journal of Immunology

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J Immunol 2013; 190:2603-2613; Prepublished online 11 February 2013;
doi: 10.4049/jimmunol.1200852
http://www.jimmunol.org/content/190/6/2603

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.

Retinoic Acid Hypersensitivity Promotes Peripheral Tolerance in Recent Thymic Emigrants

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Whereas thymic education eliminates most self-reactive T cells, additional mechanisms to promote tolerance in the periphery are critical to prevent excessive immune responses against benign environmental Ags and some self-Ags. In this study we show that murine CD4$^+$ recent thymic emigrants (RTEs) are programmed to facilitate tolerance in the periphery. Both in vitro and in vivo, naive RTEs more readily upregulate Foxp3 than do mature naive cells after stimulation under tolerogenic conditions. In RTEs, a relatively high sensitivity to retinoic acid contributes to decreased IFN-γ production, permitting the expression of Foxp3. Conversely, mature naive CD4 cells have a lower sensitivity to retinoic acid, resulting in increased IFN-γ production and subsequent IFN-γ–mediated silencing of Foxp3 expression. Enhanced retinoic acid signaling and Foxp3 induction in RTEs upon Ag encounter in the periphery may serve as form of secondary education that complements thymic education and helps avoid inappropriate immune responses. This mechanism for tolerance may be particularly important in settings where RTEs comprise a large fraction of the peripheral T cell pool, such as in newborns or after umbilical cord blood transplant. ***The Journal of Immunology*****, 2013, 190: 2603–2613.**

Mechanisms for maintaining tolerance are critical for immune homeostasis. Central tolerance via negative selection in the thymus is an important mechanism for preventing autoimmunity and results in the deletion of most thymocytes that bear strongly self-reactive αβ TCRs (1). Nevertheless, the process is imperfect, and a limited but measurable number of autoreactive T cell clones avoid deletion (owing to a low affinity of their TCR for self-Ag, a limited ability to express the self-Ag in the thymic medulla, or simply owing to chance) and successfully emigrate from the thymus to the periphery (2–8). Benign environmental Ags, such as food Ags and Ags from commensal gut flora, pose an additional problem for the immune system. Unlike genomically encoded self-Ags, environmental Ags cannot be adequately expressed by the thymic epithelium for the promotion of thymic deletion. Therefore, mechanisms to promote peripheral tolerance are essential to plug any potential "holes" in the thymic negative selection process.

CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) are the primary mediators of peripheral tolerance and act to prevent excessive immunological responses against self-Ags and benign environmental Ags (3, 9). Foxp3$^+$ "natural" Tregs arise primarily during the CD4 single-positive stage of thymic development (10). The exact signals required for the development of natural Tregs are unknown but appear to include TCR-self-peptide/MHC complex interactions in combination with CD28-CD80/86 costimulation and IL-2 (11). In contrast, induced Tregs (iTregs) arise from conventional CD4$^+$ T cells in response to Ags encountered in the periphery. The context of the Ag encounter is critical, and chronic exposure to Ag, the absence of inflammation, and elevated local levels of TGF-β and retinoic acid all promote iTreg induction (12–17). TGF-β, acting through the Smad3 signal transducer and transcriptional modulator, increases Foxp3 transcription (18), whereas retinoic acid increases Smad3 levels and binding to the Foxp3 promoter and enhancer, and it limits the inhibitory effects inflammatory cytokines such as IFN-γ and IL-21 have on Foxp3 transcription (19–21). It is thought that retinoic acid may be particularly important for the tolerance to gut Ags because gut-associated APCs are uniquely equipped to generate retinoic acid (22).

T cells that have recently emerged from the thymus, which hereafter are referred to as recent thymic emigrants (RTEs), have been shown to have a unique phenotype compared with mature naive peripheral T cells (23). Compared to their mature counterparts, CD8$^+$ RTEs demonstrate decreased cytolytic capability and proliferation in response to TCR ligation (24). CD4$^+$ RTE cells produce less IL-2 and IFN-γ than do their mature naive CD4$^+$ counterparts and have a bias toward Th2 responses (25–27). The maturation of RTEs is dependent on contact with peripheral lymphoid tissues, but neither the exact molecular signals required for maturation nor the biological purpose for this delay in function, if any, is known (28). In clinical settings in which RTEs are more abundant than mature naive cells, such as in neonates or after umbilical cord blood transplantation, deficits in T cell immunity and a tendency toward tolerance are frequently observed (29, 30).

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Received for publication March 16, 2012. Accepted for publication January 11, 2013.

This work was supported by National Institutes of Health Grants R00 HD05622 (to D.A.R.), 5 K12 HD043397-10 (to D.A.R.), and R01 AI-083757 (to D.B.L.).

D.A.R. oversaw the project and was directly responsible for the experimental design, data analysis, and figure and manuscript preparation; S.B. and D.B. were responsible for cell purification, culture, and flow cytometry; R.K. performed Luminex analysis; T.G. made the initial key observation of decreased IFN-γ production in RTEs; and D.B.L. and C.T.W. contributed key reagents and edited the manuscript.

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Abbreviations used in this article: at-RA, all-*trans* retinoic acid; iTreg, induced regulatory T cell; LPL, lamina propria lymphocyte; MLN, mesenteric lymph node; RAR, retinoic acid receptor; RTE, recent thymic emigrant; RXR, retinoid X receptor; Treg, regulatory T cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200852

We hypothesized that the functional immaturity of RTEs might serve to promote peripheral tolerance. Although thymic education is primarily responsible for maintaining tolerance to self-Ags, a programmed delay in the capacity of RTEs to mount strong T cell responses might provide an opportunity for T cell education in the periphery. This second opportunity for T cell education could facilitate the induction of tolerance in the rare self-reactive T cells that escape thymic deletion and promote tolerance to common environmental Ags, including those derived from food and gut commensal flora.

Through the use of NG-BAC reporter mice in which RTEs can be identified as GFP^+ cells in the peripheral lymphoid tissues, we show in this study that GFP^+ naive RTEs more readily upregulate Foxp3 across a wide range of conditions both in vitro and in vivo as compared with GFP^- mature naive $CD4^+$ T cells. Compared to mature naive $CD4^+$ T cells, RTEs express more retinoic acid receptors α and β (RARα and RARβ), display increased sensitivity to retinoic acid, and more readily upregulate Foxp3 under tolerogenic conditions. In contrast, the mature naive $CD4^+$ T cells produce more inflammatory cytokines (including IFN-γ) than do RTEs, and these cytokines suppress Foxp3 induction in mature naive T cells in an autocrine and paracrine fashion. Taken together, these results suggest that RTEs more readily undergo peripheral tolerance, which may serve as an additional layer of prevention against the induction of inappropriate immune responses by T cells that recognize common environmental Ags or that are self-reactive and escape thymic deletion.

Materials and Methods

Mice

NG-BAC transgenic mice (expressing GFP under regulation of the Rag2 gene) (31) that had been previously backcrossed for more than eight generations onto the BALB/c or C57BL/6 background were obtained from Dr. David Lewis (Stanford University) and Dr. Pamela Fink (University of Washington), respectively. These were further crossed with BALB/c Thy1.1 congenic mice, $Rag2^{-/-}$ DO11.10 TCR transgenic mice (specific for the $OVA_{323–339}$ peptide on $I\text{-}A^d$) (32), or with IFN-$\gamma^{-/-}$ OT-II TCR transgenic mice (specific for the $OVA_{323–339}$ peptide on $I\text{-}A^b$) (33). All mice were maintained under specific pathogen-free conditions and used at 4–12 wk of age in accordance with the approved protocols and guidelines of the Institutional Animal Care and Use Committees of the University of Alabama at Birmingham and of Stanford University.

Cell preparation, sorting, and flow cytometry

Single-cell suspensions were prepared from femoral, axillary, cervical, and mesenteric lymph nodes and spleens. $CD4^+$ T cells were first enriched by negative selection with a mixture of mAb-labeled paramagnetic beads (Miltenyi Biotec) and were then FACS-sorted. RTEs were sorted as $CD4^+CD45RB^{hi}CD25^-GFP^+$ cells. Mature naive cells were sorted as $CD4^+CD45RB^{hi}CD25^-GFP^-$ cells. The top 15% of cells in the GFP channel were collected as GFP^+ RTEs, and the bottom 15% were collected as GFP^- mature naive cells. In some experiments, $CD62L^{hi}$ was substituted for $CD45RB^{hi}$ as a marker for naivety, and this alternative sorting strategy led to similar results. Abs specific for CD4, CD25, CD62L, CD90.1 (Thy1.1), CD90.2 (Thy1.2), NK1.1, $I\text{-}A^d$, CD11c, CD44, CCR9, CD103, CD45.1, and IFN-γ were purchased from BD Biosciences and were used as biotin, PE-Cy7, allophycocyanin, PE, FITC, or PerCP-Cy5.5 conjugates for staining. Anti-Foxp3 was purchased from eBioscience. Unlabeled anti-RARα (Santa Cruz Biotechnology) and anti-RARβ (Novus Biologicals) Abs were labeled prior to use with a Zenon rabbit IgG labeling kit (Invitrogen). For all data shown, dead cells were excluded using Live/Dead fixable near-IR stain (Invitrogen).

Adoptive transfer experiments

For polyclonal adoptive transfer experiments, $CD4^+CD45RB^{hi}CD25^-$ T cells from $Thy1.1^+$ NG-BAC mice were sorted into GFP^+ (RTE) and GFP^- (mature naive) fractions and transferred into BALB/c recipients by i.v. injection. Two weeks later, peripheral lymph nodes, mesenteric lymph nodes (MLNs), spleens, and intestinal tissues were harvested. For spleens and peripheral lymph nodes, $CD4^+$ T cells were enriched using anti-CD4 paramagnetic beads (Miltenyi Biotec) prior to staining. For lamina propria lymphocytes (LPLs), the intestines were inverted on a glass pipette and then stripped of epithelium by successive 5 mM Na-EDTA washes. The tissue was minced, digested for 30 min with 0.1 Wünsch unit/ml Liberase (Roche), and the digest was overlayed on a 40/80% Percoll gradient. After centrifugation, the cells at the interface were collected and used for mAb staining.

Oral tolerance was induced using a protocol similar to Sun et al. (22). Briefly, 1×10^6 GFP^+ RTEs or GFP^- mature naive $CD4^+$ T cells were purified from $Thy1.2^+$ NG-BAC $Rag^{-/-}$ DO11.10 mice and transferred by i.v injection into BALB/c $Thy1.1^+$ congenic recipient mice. The recipients were placed on a diet of conventional mouse chow and sterile drinking water that contained 1% OVA (Sigma-Aldrich). After 5 d, MLNs and spleens were collected, and the Foxp3 expression of the transferred $Thy1.2^+$ cells was assessed by flow cytometry. In the cotransfer experiments, $CD4^+CD25^-$ L-$selectin^{hi}GFP^+$ RTE or GFP^- mature naive cells were sorted from OT-II NG-BAC mice that were $CD45.1^{+/-}$ or $CD45.1^{+/+}$, respectively. The cells were then mixed at a 3:1 ratio and 1×10^6 total cells were transferred to $CD45.2^{+/+}$ WT recipients. A 3:1 RTE to mature cell ratio was chosen to minimize suppression of Foxp3 expression in the RTEs by IFN-γ produced by the mature cells. In some experiments, mice received 500 μg neutralizing anti–IFN-γ Ab (clone XMG1.2; University of Alabama at Birmingham Hybridoma Center) or a rat IgG1 isotype control via i.p injection on day 1 and day 3. The experiments were also repeated in C57BL/6 (CD45.2) mice using transferred cells from $CD45.1^+$ NG-BAC OT-II mice on an IFN-$\gamma^{-/-}$ or IFN-$\gamma^{+/+}$ background.

Tissue culture

For all experiments, cells were cultured at 37°C under 7% CO_2 in complete IMDM supplemented with 10% FBS containing physiologic quantities of retinoic acid, 25 mM HEPES, 2 mM L-glutamine, 110 μg/ml sodium pyruvate, nonessential amino acids, penicillin/streptomycin, and 50 μM 2-ME. For neutral conditions, sorted cells (10^5 cells/well) were stimulated for 5–7 d with anti-CD3/anti-CD28 Dynal T cell stimulator beads (Invitrogen) at a 1:1 cell/bead ratio in a 96-well plate in media alone. For Treg conditions, recombinant human TGF-β (5 ng/ml) (R&D Systems) was added. Some cultures were also treated with all-*trans* retinoic acid (at-RA; Sigma-Aldrich) at the indicated concentrations. For in vitro cytokine neutralization, anti–IFN-γ (clone XMG1.2; University of Alabama at Birmingham Hybridoma Center), anti–IL-21 (R&D Systems), or rat IgG1 isotype control (eBioscience) were added to a final concentration of 10 μg/ml. For IFN-γ–mediated suppression of Foxp3, recombinant murine IFN-γ (R&D Systems) was added at 10 U/ml. For peptide stimulation, $CD4^+$ T cells were cultured with APCs (irradiated T cell–depleted BALB/c splenocytes) and 5 μg/ml $OVA_{323–339}$ peptide (Anaspec). For intracellular cytokine analysis, cells were restimulated on days 5–7 with PMA (10 ng/ml), ionomycin (0.5 μg/ml), and brefeldin A (5 μg/ml) (all from Sigma-Aldrich) and then fix and stained using the Cytofix/Cytoperm buffer set (BD Biosciences). For Foxp3 staining, cells were stained with anti-Foxp3 Ab in Foxp3 buffer (eBioscience) per the manufacturer's instructions.

For suppression studies, sorted GFP^+ RTEs and GFP^- mature $CD4^+$ T cells were stimulated with anti-CD3/CD28 microbeads and TGF-β (5 ng/ml). After 5 d the cells were washed, counted, and added at a ratio of 2:1 to CFSE-labeled Thy1.1 congenic responder cells. The T cells were then stimulated with APCs and 5 μg/ml anti-CD3. On day 3 of coculture, proliferation of the $Thy1.1^+$ cells was assessed by flow cytometry.

For cell mixing experiments, GFP^+ RTEs and GFP^- mature naive $CD4^+$ T cells were sorted from NG-$BAC^+Thy1.1^+$ and NG-$BAC^+Thy1.2^+$ mice. The sorted $Thy1.2^+$ mature naive cells and $Thy1.2^+$ RTEs were mixed with $Thy1.1^+$ RTE and $Thy1.1^+$ mature naive cells, respectively, at various ratios (1:0, 1:1, 1:3) and stimulated with T cell activator beads at a 1:1 cell/bead ratio in the presence of recombinant human TGF-β (5 ng/ml). On day 5, cells were stained for Thy1.1, Thy1.2, and intracellular Foxp3 and analyzed by flow cytometry.

Cytokine measurements

Multiplex bead array analysis of cytokines in the tissue culture supernatants of RTEs and mature naive $CD4^+$ T cells was performed with a Milliplex mouse cytokine kit (Millipore). Briefly, tissue culture supernatants were collected from RTE and mature naive cell cultures activated with anti-CD3/anti-CD28 beads or irradiated APCs and $OVA_{323–339}$ peptide with media alone or with TGF-β added (5 ng/ml). Supernatants were collected after 24–96 h stimulation and stored at −80°C until they could be analyzed by multiplex analysis per the manufacturer's protocol. Cytokine levels were quantified with a Bio-Plex analyzer (Bio-Rad).

Real-time PCR

Total RNA was isolated from GFP^+ RTEs and GFP^- mature naive $CD4^+$ T cells stimulated for 48 h with anti-CD3/CD28 beads and 5 ng/ml TGF-β

using TRIzol (Invitrogen), and cDNA was prepared using a SuperScript III first-strand synthesis kit (Invitrogen) per the manufacturer's instructions. Real-time PCR was performed with a Bio-Rad iCycler system. Primer pairs used were as follows: *Rara* (5′-TCCGACGAAGCATCCAGAA-3′, 5′-GGTTCCGGGTCACCTTGTT-3′), *Rarb* (5′-CAGTGAGCTGGCCACCAAGT-3′, 5′-GCGATGGTCAGACCTGTGAA-3′), *Rarg* (5′-GTCAGCTCCTGTGAAGGCTGC-3′, 5′-GTTTCGATCGTTCCTTACAG-3′), *Rxra* (5′-TAGTCGCAGACATGGACACC-3′, 5′-GTTGGAGAGTTGAGGGACGA-3′), *Rxrb* (5′-GCACAGAAACTCAGCCCATT-3, 5′-CATCCTCATGTCACGCATTT-3′), *Rxrg* (5′-GCCTGGGATTGGAAATATGA-3′, 5′-ACACCGTAGTGCTTCCCTGA-3′), and 18S sRNA (5′-GCCGCTAGAGGTGAAATTCTTG-3′, 5′-CATTCTTGGCAAATGCTTTCG-3′).

Data analysis

All flow cytometric data were initially collected on an LSRII flow cytometer (BD Biosciences), and the data were later analyzed with FlowJo FACS analysis software (Tree Star). Graphing and statistical analysis, including SEM and *t* test calculations, were performed with Prism software (GraphPad Software).

Results

Foxp3 is preferentially upregulated in RTEs after polyclonal transfer

We began by determining whether polyclonal populations of RTE naive Foxp3$^-$CD4$^+$ T cells more readily convert to a Foxp3$^+$ phenotype than do mature naive Foxp3$^-$CD4$^+$ T cells after being transferred to unmanipulated BALB/c mice. In NG-BAC Rag2-GFP reporter mice, RTEs in the peripheral tissues are marked by residual GFP fluorescence due to recent *Rag2* promoter activity during intrathymic maturation (24, 31), and thus GFP$^+$ RTEs can be separated by cell sorting from GFP$^-$ mature naive cells in which the GFP signal has decayed as the cells circulate in the periphery. Polyclonal CD4$^+$CD45RB^{hi}CD25$^-$ naive cells from NG-BAC Thy1.1 congenic mice were sorted into GFP$^+$ RTE and GFP$^-$ mature naive fractions and then transferred to BALB/c mice at 2×10^6 cells/recipient. Two weeks later, CD4$^+$ cells were purified from the spleens, MLNs, and intestinal lamina propria and stained for Thy1.1 (to identify transferred cells) and Foxp3. Prior to transfer, the starting GFP$^+$CD25$^-$ RTE and GFP$^-$CD25$^-$ mature naive CD4$^+$ cells had small but detectible subpopulations that were Foxp3$^+$ (Fig. 1A). By 2 wk, the percentage of Foxp3$^+$ cells had increased in both transferred populations. However, this increase was significantly greater in transferred RTEs than in transferred mature naive cells (Fig. 1B), particularly for the cells recovered from the lamina propria (14.2 versus 8.9% Foxp3$^+$, $p < 0.001$) despite the fact that the transferred RTEs started with fewer Foxp3$^+$ cells (0.6% for RTE and 1.4% for mature naive, $p < 0.05$) (Fig. 1A). Significant differences between RTE and mature naive cells in the numbers of transferred cells recovered relative to the total CD4 cells were not observed (recovered cells as percentage of spleen CD4 cells = 0.5 ± 0.2% RTE versus 0.4 ± 0.3% mature naive, as percentage of MLN CD4 cells = 0.4 ± 0.1% RTE versus 0.5 ± 0.1% mature naive, and as percentage of LPL CD4 cells = 1.4 ± 0.20% RTE versus 1.4 ± 0.5% mature naive; p = NS for all). Also, similar results were obtained when L-selectin (CD62L) was used instead of CD45RB as a marker of naivety although recovery of both cell types from the lamina propria tended to be lower when L-selectin was used, perhaps due to preferential lymph node homing (data not shown). Taken together, these results suggest that RTEs might convert to a Foxp3$^+$ phenotype in the periphery more readily than mature naive CD4$^+$ T cells, particularly in GALTs.

Ag-specific RTEs preferentially upregulate Foxp3 in vitro and in vivo

We next assessed the ability of GFP$^+$CD4$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cells to upregulate Foxp3 in vitro under varying concentrations of at-RA and TGF-β. at-RA is thought to be particularly important in the induction of iTregs in the GALT and has been shown to act synergistically with TGF-β in inducing iTregs in vitro (16, 19, 22, 34, 35). To control for any potential differences in TCR specificities between RTEs and mature naive cells and to minimize initial contamination with Foxp3$^+$ cells, NG-BAC mice were crossed with Rag2$^{-/-}$ DO11.10 TCR transgenic mice to generate double transgenic animals in which all CD4$^+$ T cells were specific for chicken egg OVA peptide. Similar to other TCR transgenic strains, the presence of the OVA-specific TCR on a Rag2-deficient background results in a uniform CD4$^+$ T cell population that possesses minimal Foxp3 expression at baseline and provides a convenient source of naive Foxp3$^-$CD4$^+$ T cells (36). We first compared the induction of Foxp3 expression in purified GFP$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cells from these mice after stimulation of 10^5 cells in vitro for 5 d with anti-CD3/anti-CD28 mAb-conjugated beads in the presence of TGF-β with and without at-RA (0.1 nM) (Fig. 2A). After 4 d, total cell numbers were similar ($2.8 \pm 0.3 \times 10^5$ RTE versus $2.3 \pm 0.5 \times 10^5$ mature cells for 0.1 nM at-RA; p = NS), but RTEs converted to a Foxp3$^+$ phenotype more readily than did mature cells. This held true across a wide range of TGF-β and at-RA concentrations (Fig. 2B, 2C), and the difference between RTE and mature cells was saturable at high doses of at-RA but not TGF-β. Addition of TGF-β was sufficient to drive Foxp3 expression with the baseline levels of retinoic acid present in the media from serum. at-RA alone could not induce Foxp3 expression without addition of exogenous TGF-β, suggesting a synergistic roles of at-RA and TGF-β. Foxp3 expression was associated with suppressive function, and RTE-derived iTregs were as effective as iTregs derived from mature naive cells in suppressing proliferation in vitro (Fig. 2D). Increased Foxp3 expression was not an artifact of the DO11.10 TCR transgene nor was it unique to the BALB/c background, as polyclonal populations from BALB/c NG-BAC and C57BL/6 NG-BAC mice behaved similarly.

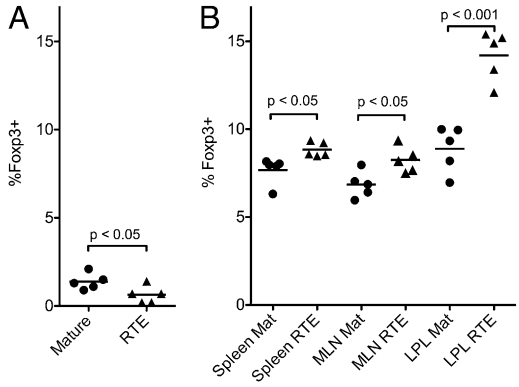

FIGURE 1. Peripheral acquisition of Foxp3 expression in RTEs after adoptive transfer. (**A**) Starting percentages of Foxp3$^+$ cells in sorted RTE and mature naive CD4$^+$ cells prior to transfer. Polyclonal populations of naive CD4$^+$CD25$^-$CD45RB^{hi} cells from NG-BAC Thy1.1 mice were sorted into GFP$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cell populations. In each of five separate sorts, Foxp3$^+$ cells were rarer in sorted CD25$^-$ RTEs than in CD25$^-$ mature naive cells prior to transfer (0.6 versus 1.4%, $p < 0.05$). (**B**) Percentages of Foxp3$^+$ cells in the transferred RTE and mature cells recovered from each tissue. After sorting, 2×10^6 RTEs or mature naive Thy1.1$^+$ CD4$^+$ cells were adoptively transferred into BALB/c hosts (n = 5/group). Two weeks later, CD4$^+$ cells from spleens, MLNs, and the intestinal LPLs were isolated and stained for Foxp3 and Thy1.1 to identify the transferred RTE or mature (Mat) cells. Bars indicate mean Foxp3 percentages with p values (Student t test) as indicated. The results are representative of five similar experiments.

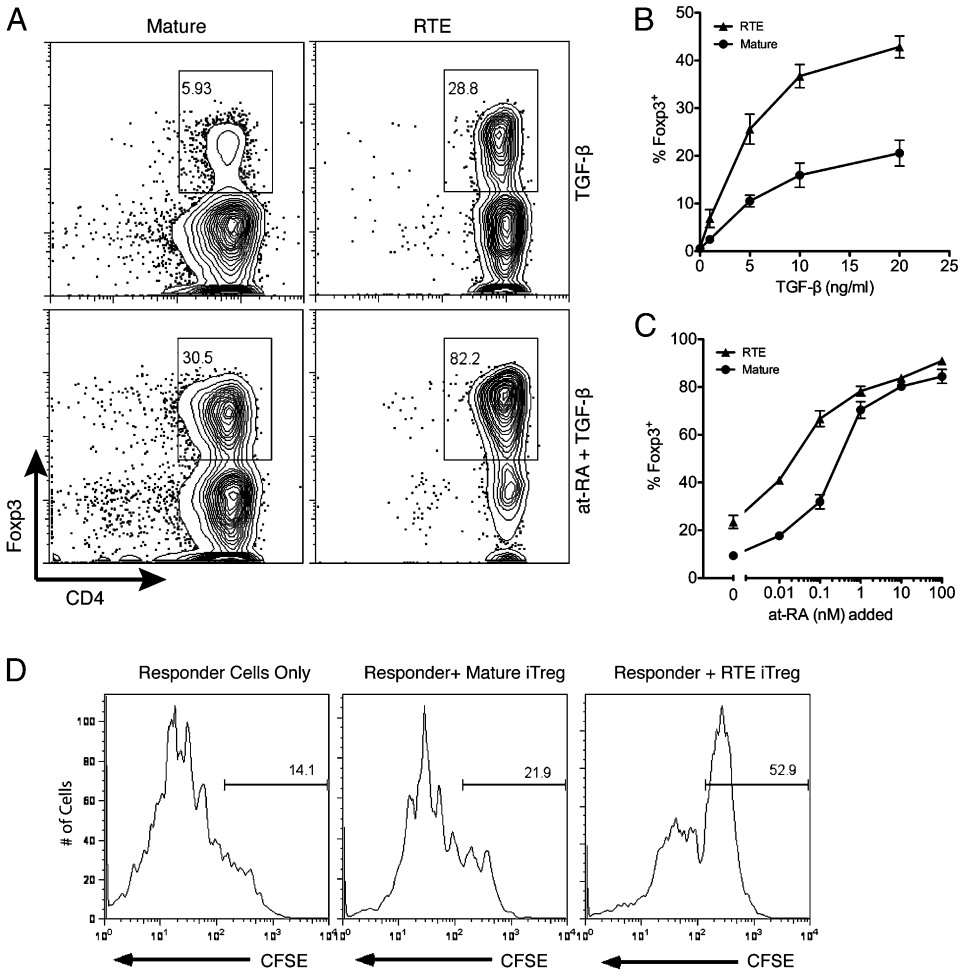

FIGURE 2. Increased Foxp3 induction in RTEs in vitro. (**A**) Flow cytometric analysis of Foxp3 expression in cultured RTE and mature naive cells. GFP$^-$ mature naive and GFP$^+$ RTE cells were sorted from NG-BAC Rag2$^{-/-}$ DO11.10$^+$ mice and stimulated in vitro with anti-CD3/anti-CD28 microbeads and 5 ng/ml TGF-β with or without 0.1 nM at-RA added as indicated and then analyzed on day 5 for Foxp3 expression. The percentages of Foxp3$^+$ cells are indicated. (**B**) Graph of the percentage of Foxp3$^+$ cells in mature naive CD4$^+$ T cells (●) and RTEs (▲) across a range of TGF-β concentrations with no at-RA added above the basal levels present from serum in the media. Plotted are the means ± SD of triplicate samples for each condition. Similar data were obtained in three separate experiments. (**C**) Graph of the percentage of Foxp3$^+$ cells in mature naive CD4$^+$ T cells (●) and RTEs (▲) across a range of at-RA concentrations with 5 ng/ml TGF-β. Plotted are the means ± SD of triplicate samples for each condition. Similar data were obtained in five separate experiments. (**D**) RTE-derived iTregs are suppressive in vitro. CFSE-labeled responder cells were cocultured with APCs, anti-CD3 (5 μg/ml), and either RTE- or mature T cell–derived iTregs at a ratio of 1:2. Proliferation of the responder cells was assessed by flow cytometry on day 3. Data are representative of three separate experiments.

To determine whether RTEs would also be easier to render tolerant in vivo, we adapted an oral tolerance model to our system. GFP$^+$ RTEs and GFP$^-$ mature naive cells were sorted from NG-BAC Rag2$^{-/-}$ DO11.10 Thy1.2$^+$ mice and then transferred into Thy1.1$^+$ congenic BALB/c mice. The recipient animals were placed on a conventional diet supplemented with 1% OVA in the drinking water. As shown in Fig. 3A and 3B, after 5 d a significantly higher fraction of the transferred RTEs were Foxp3$^+$ in both the spleen (9.8 versus 3.5%; $p < 0.05$) and MLN (42 versus 20%; $p < 0.01$) when compared with transferred mature naive CD4$^+$ T cells. Significant differences were not observed in total recovered RTE and mature cells relative to total CD4 cells (recovered cells as percentage of total MLN CD4 cells = 0.3 ± 0.2% RTE versus 0.4 ± 0.3% mature, p = NS; as percentage of total spleen CD4 cells = 0.2 ± 0.1% RTE versus 0.4 ± 0.3% mature, p = NS).

The increased percentages of Foxp3$^+$ cells observed after transfer of RTEs could be due to increased conversion of RTEs to a Foxp3$^+$ phenotype or due to poor survival of Foxp3$^-$ RTE cells in vivo or both. As described above, cell numbers recovered after transfer did not appear to be significantly different; however, we wanted to directly test the relative importance of these two mechanisms. We therefore performed a competitive transfer experiment by cotransferring allelically marked CD45.1$^{+/-}$ RTE and CD45.1$^{+/+}$ mature cells together into CD45.2$^{+/+}$ recipient animals and repeating the oral tolerance experiment (Fig. 3C, 3D). When compared side by side in the same animal, similar numbers of total RTEs and mature cells were recovered but the RTEs again had higher numbers of Foxp3$^+$ cells, indicating that conversion to a Foxp3$^+$ phenotype is the dominant mechanism.

IFN-γ production by mature naive CD4$^+$ T cells inhibits Foxp3 induction in vitro and in vivo

To begin to dissect the mechanisms for the observed differences between RTEs and mature naive CD4$^+$ T cells, we next performed a mixing experiment with polyclonal populations of allelically marked RTEs and mature naive cells. Sorted CD25$^-$GFP$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cells from NG-BAC Thy1.2 BALB/c mice were mixed with sorted mature naive or RTE CD4$^+$ T cells, respectively, from NG-BAC Thy1.1 mice in varying ratios. The resulting cell mixtures were stimulated with anti-CD3/anti-

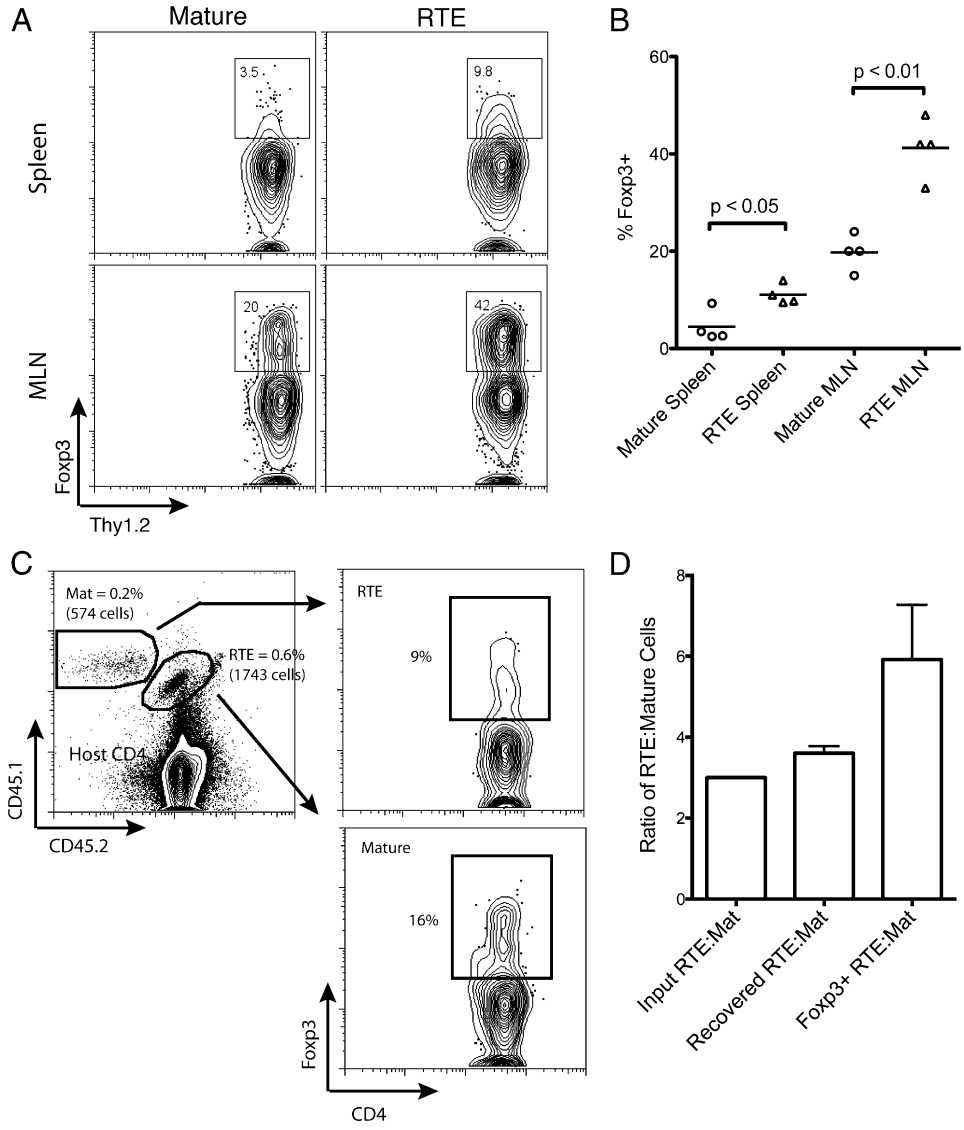

FIGURE 3. Increased Foxp3 expression in RTEs compared with mature naive CD4$^+$ T cells in Ag-specific oral tolerance. In (**A**) and (**B**), Thy1.2$^+$ CD4$^+$ cells from NG-BAC Rag$^{-/-}$ DO mice were sorted into GFP$^-$ mature naive and GFP$^+$ RTE fractions, and 1×10^6 cells were transferred to groups ($n = 4$) of Thy1.1 congenic BALB/c recipients. After 5 d on a diet that included 1% OVA in the drinking water, spleens and MLNs were collected, and the Thy1.2$^+$ transferred cells were analyzed by flow cytometry for Foxp3 expression. In (**C**) and (**D**), GFP$^+$ RTEs from CD45.1$^{+/-}$ OT-II mice were mixed at a ratio of 3:1 with GFP$^-$ mature cells from CD45.1$^{+/+}$ OT-II mice and 1×10^6 total cells were transferred to CD45.2$^{+/+}$ WT recipients. The recipients were placed on an OVA diet as above and MLN cells were analyzed by flow cytometry on day 5. (A) Representative FACS plots of Foxp3 expression in recovered Thy1.2$^+$ cells from spleens and mesenteric lymph nodes as indicated. (B) Summarized oral tolerance results from one experiment. Bars indicate the mean for each group with p values (Student t test) as indicated. Similar results were obtained in three separate experiments. (C) Representative FACS plots showing recovery of allelically marked RTE and mature Ag-specific cells after cotransfer. (D) Summary plot showing the ratios of allelically marked RTE and mature cells in transferred and recovered cells. RTEs showed equivalent survival to mature cells and a trend toward increased conversion to a Foxp3$^+$ phenotype with the ratio of total RTE/mature cells recovered matching the 3:1 input ratio and the ratio of recovered Foxp3$^+$RTE/Foxp3$^+$ mature cells equaling 6:1 ($p = 0.1$, $n = 4$ animals). Shown are the mean ratios ± SD.

CD28 beads with 5 ng/ml TGF-β for 5 d. Although mature naive cells inhibited Foxp3$^+$ induction in RTEs at high concentrations, the conversion of mature naive cells to the Foxp3$^+$ phenotype was largely unaffected by the presence of RTEs (Fig. 4) ($p < 0.05$ for trend comparison). This suggested that factors from the mature cells might act in an autocrine or paracrine fashion to inhibit Foxp3 induction.

A number cytokines, including IFN-γ, IL-4, IL-6, IL-21, and TNF-α, have been reported to prevent Foxp3 induction (37–41). To determine whether RTEs and mature naive cells differ in cytokine production, we stimulated sorted GFP$^+$ and GFP$^-$CD4$^+$ T cells from NG-BAC Rag2$^{-/-}$ DO11.10 mice with anti-CD3/anti-CD28 beads (Fig. 5A) or OVA peptide and APCs (Fig. 5B) in media alone (neutral conditions) with or without 5 ng/ml TGF-β added (Treg conditions) and measured the cytokine production by multiplex cytokine analysis. Compared to the mature naive cells, RTEs produced much lower levels of IFN-γ, IL-17, and TNF-α but higher levels of IL-4 under both neutral and Treg conditions, suggesting a less inflammatory phenotype. Similar results were obtained when RTEs were stimulated with APCs, with increased levels of IL-4 and lower levels of IFN-γ and IL-17 being detected in the culture supernatants.

To determine whether the differential cytokine production between RTEs and mature naive CD4$^+$ T cells contributed to the differences in Foxp3 induction, we stimulated sorted GFP$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cells under Treg conditions in the

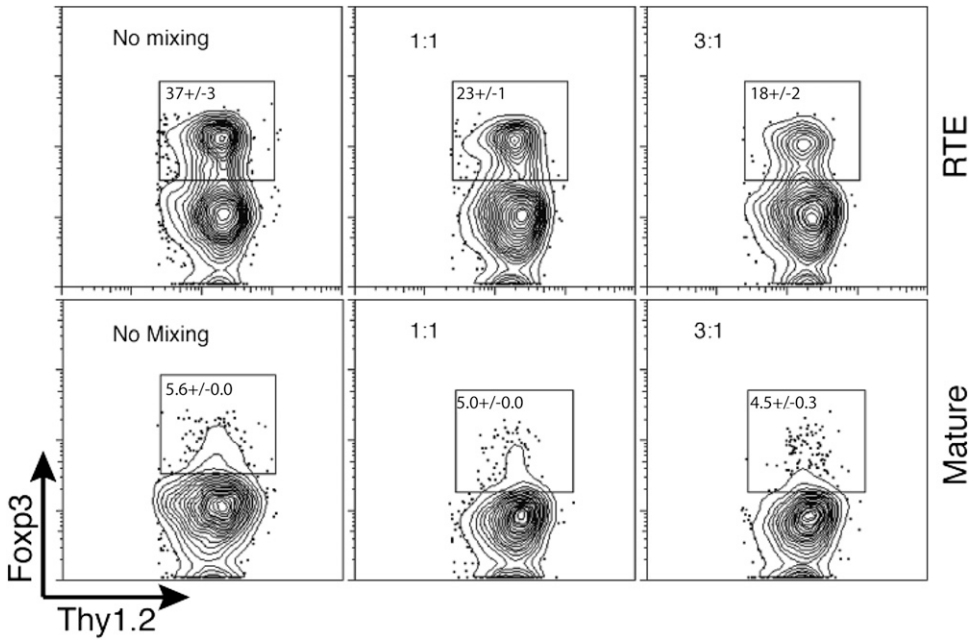

FIGURE 4. Mature naive CD4$^+$ T cells reduce Foxp3 induction in RTEs in mixed cultures. CD4$^+$CD62L$^+$CD25$^-$ RTE and mature naive cells from NG-BAC Thy1.1$^+$ and NG-BAC Thy1.2$^+$ animals were sorted. Thy1.2$^+$ RTEs or Thy1.2$^+$ mature naive cells were stimulated with anti-CD3/anti-CD28 microbeads and 5 ng/ml TGF-β. Cells were stimulated either alone or mixed with the indicated ratios of Thy1.1$^+$ mature/Thy1.2$^+$ RTE cells (*top panels*) or Thy1.1$^+$ RTE/Thy1.2$^+$ mature cells (*bottom panels*). On day 5, the percentages of Foxp3$^+$Thy1.2$^+$ cells were assessed by flow cytometry. The percentages of Foxp3$^+$ cells ± SD for one experiment are shown. Data are representative of four separate experiments.

presence of recombinant IFN-γ or neutralizing Abs to IFN-γ. The presence of exogenous IFN- γ completely abrogated Foxp3 induction after the stimulation of RTEs (Fig. 6A) (24 versus 0.7% Foxp3$^+$; $p < 0.001$), whereas the presence of neutralizing Abs against IFN-γ had little effect on the Foxp3 levels in RTEs, which produce minimal amounts of IFN-γ (27 versus 31% Foxp3$^+$ for RTE versus RTE plus anti–IFN-γ; p = NS) (Fig. 6B). However, the presence of anti–IFN-γ dramatically increased Foxp3 expression in stimulated mature naive T cells to levels equivalent or higher than those seen in RTEs (38 versus 13% Foxp3$^+$ for mature versus mature plus anti–IFN-γ; $p < 0.001$). This suggests that inflammatory cytokines, particularly IFN-γ, produced by mature naive CD4$^+$ T cells even under Treg conditions are responsible for autocrine inhibition of Foxp3 expression in mature naive cells (Fig. 6B) and paracrine inhibition of RTE Foxp3 expression in mixed cultures (Fig. 4).

To determine whether IFN-γ acts in an autocrine fashion to inhibit Foxp3 induction in mature naive CD4$^+$ T cells in vivo, we next repeated the oral tolerance experiments using sorted cells from NG-BAC CD45.1 congenic mice that had been crossed with IFN-γ$^{-/-}$ OT-II mice. Sorted OVA-specific GFP$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cells were obtained from IFN-γ$^{-/-}$ or IFN-γ$^{+/+}$ NG-BAC OT-II CD45.1$^+$ mice, and transferred to C57BL/6 CD45.2 congenic recipients. After 5 d with OVA in the drinking water, the MLNs were collected, and the recovered transferred CD45.1$^+$ cells were analyzed for Foxp3 expression. As before, IFN-γ$^{+/+}$ RTEs converted to Foxp3$^+$ cells at a higher frequency than did mature naive IFN-γ$^{+/+}$ CD4$^+$ T cells ($p < 0.01$) (Fig. 6C). However, no significant difference was seen between RTE and mature naive cells when IFN-γ$^{-/-}$ cells were used, confirming a critical role for IFN-γ (Fig. 6C). Recovery of the transferred RTEs as a percentage of total MLN CD4 cells was not significantly different from mature cells (1.3 ± 0.3% IFN-γ$^{+/+}$ RTE versus 1.0 ± 0.2% IFN-γ$^{+/+}$ mature naive, p = NS; 1.9 ± 0.4% IFN-γ$^{-/-}$ RTE versus 1.8 ± 0.3% IFN-γ$^{-/-}$, p = NS). Similar results were obtained in BALB/c Thy1.1 congenic mice using neutralizing anti–IFN-γ or isotype control Ab after transfer of sorted RTE or mature naive cells from NG-BAC Rag$^{-/-}$DO11.10 mice.

Differential sensitivity to at-RA between RTEs and mature naive cells

The dose-response curve of Foxp3 induction at varying concentrations of at-RA (Fig. 2C) suggested that RTEs might have heightened sensitivity to at-RA, perhaps due to increased receptor expression, as the difference between RTEs and mature naive cells was saturable at high at-RA doses. To test the existence of increased retinoic acid signaling in RTEs, we next measured the induction of additional retinoic acid–dependent genes. The expression of CD103 and CCR9 in activated CD4 T cells has been reported to be dependent on retinoic acid signaling (16). Therefore, we activated GFP$^+$ RTEs and GFP$^-$ mature naive cells in the presence of TGF-β and varying concentrations of at-RA and assessed CD103, CCR9, and Foxp3 expression. Even when stimulated with low concentrations of at-RA, most CD4$^+$ RTEs expressed CD103 (Fig. 7A). However, much higher concentrations were needed to induce the expression of CD103 on mature naive CD4$^+$ T cells. As before, the expression of Foxp3 was significantly greater after low dose at-RA and TGF-β stimulation in RTEs than in mature naive cells. Similarly, lower concentrations of at-RA were needed to induce CCR9 expression in RTEs than in mature cells (Fig. 7A). Thus, three different retinoic acid–dependent genes (Foxp3, CD103, CCR9) are all more readily induced on RTEs, suggesting that RTEs are more sensitive than mature naive CD4$^+$ T cells to at-RA.

Retinoic acid signaling is mediated by the retinoic acid receptors, RARα, RARβ, and RARγ, and the retinoid X receptors, RXRα, RXRβ, and RXRγ. To determine whether differential expression of these receptors could be responsible for the differences in responsiveness to at-RA between RTEs and mature naive cells, we assessed receptor expression by real-time PCR in sorted RTEs and mature naive cells. After the cells were stimulated for 48 h with CD3/CD28 microbeads and TGF-β (5 ng/ml), the expression

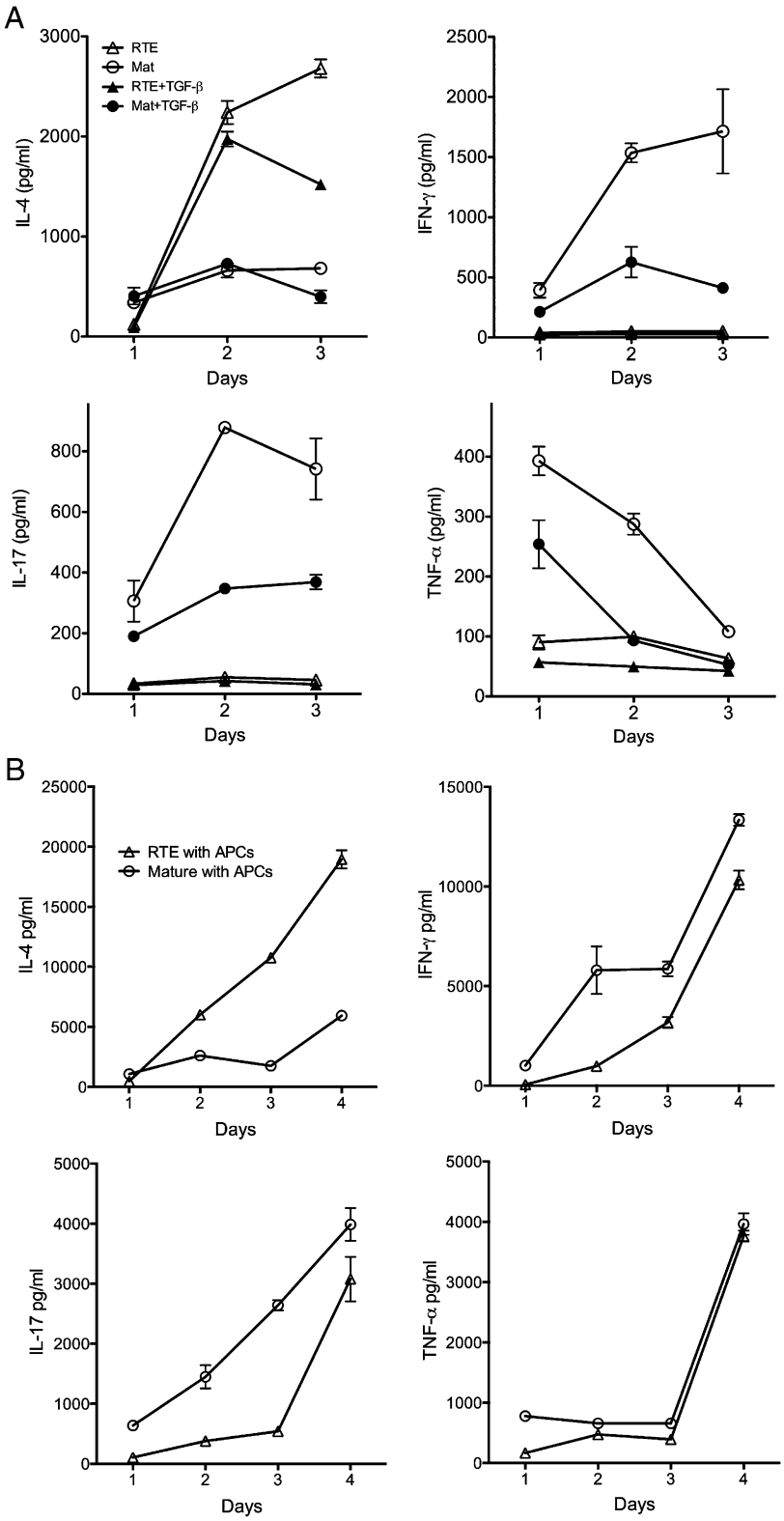

FIGURE 5. Cytokine production by stimulated RTEs and mature naive cells. (**A**) Sorted $CD4^+$ L-selectin$^+$GFP$^+$ RTEs and GFP$^-$ mature naive T cells were stimulated in vitro with anti-CD3/anti-CD28 microbeads in media alone or with 5 ng/ml TGF-β added. Supernatants were collected at the indicated times, and cytokines were measured by multiplex bead array. △, RTE $CD4^+$ stimulated in media; ▲, RTE $CD4^+$ stimulated in media plus TGF-β; ○, mature naive $CD4^+$ T cells stimulated in media; and ●, mature naive cells stimulated in media with TGF-β. (**B**) Sorted RTE (△) and mature naive cells (○) were stimulated with irradiated APCs and $OVA_{323–339}$ peptide. The data represent the mean of triplicate samples ± SEM. Similar results were obtained in three separate experiments.

levels of the RARs were higher in RTEs than in mature naive cells ($p < 0.05$ for RARα, RARβ, and Foxp3) (Fig. 7B). The difference in RARβ levels was particularly striking, with expression being 15-fold higher in RTEs than in mature naive cells. RXRγ was not detected in either population. To validate the real-time PCR data, we also analyzed RARα and RARβ expression by intracellular staining and flow cytometry (Fig. 7C). In parallel with the real-time data, both RTEs and mature cells upregulated RARα and RARβ after activation with TGF-β, but expression of RARα and especially RARβ was higher in RTEs.

One mechanism by which at-RA has been shown to promote Foxp3 is via the suppression of IFN-γ transcription (20). We predicted that in the absence of IFN-γ, Foxp3 expression would be restored in mature cells but that RTEs would continue to preferentially express other at-RA–dependent (but IFN-γ–independent) genes such as CD103 and CCR9. Indeed, activation of sorted RTE

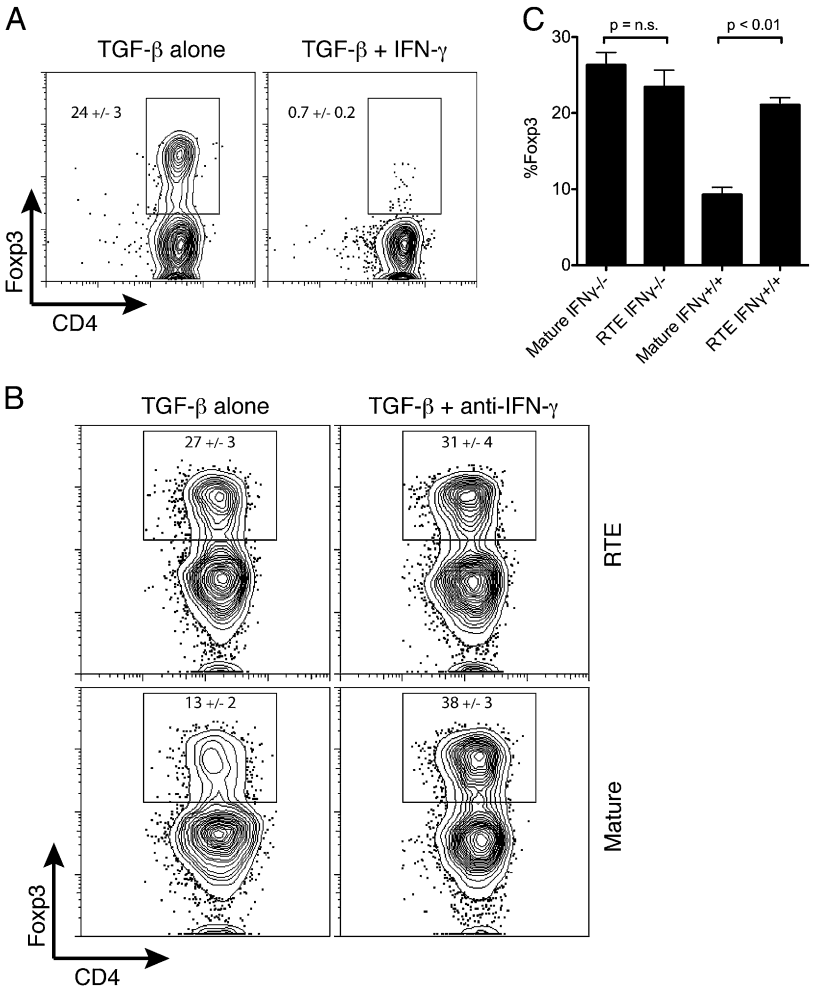

FIGURE 6. Role of IFN-γ in differential Foxp3 induction in RTEs and mature naive cells. (**A**) Exogenous IFN-γ suppresses Foxp3 induction in RTEs. GFP$^+$ RTEs were sorted from NG-BAC Rag2$^{-/-}$ DO11.10$^+$ mice and stimulated with anti-CD3/anti-CD28 microbeads and TGF-β (5 ng/ml) alone or with rIFN-γ (10 U/ml) added. Foxp3 expression was assessed on day 5 by flow cytometry. The percentages of Foxp3$^+$ cells ± SD are indicated. Data are representative of three separate experiments. (**B**) IFN-γ neutralization restores Foxp3 expression in mature naive cells in vitro. Sorted GFP$^+$ RTE or GFP$^-$ mature naive CD4 T cells from NG-BAC Rag$^{-/-}$DO$^+$ mice were stimulated as in (A) with or without the addition of anti–IFN-γ (10 μg/ml) as indicated. The percentage of Foxp3$^+$ cells ± SD is indicated. Similar results were obtained in three separate experiments. (**C**) IFN-γ deficiency restores Foxp3 expression in mature naive CD4$^+$ T cells during oral tolerance induction in vivo. Naive mature and RTE CD4$^+$ T cells were sorted from IFN-γ$^{-/-}$ or IFN-γ$^{+/+}$ NG-BAC OT-II CD45.1$^+$ mice and transferred to groups of C57BL/6 CD45.2 congenic mice (n = 5), which received 1% OVA in their drinking water for 5 d. MLNs were collected, and the transferred CD45.1$^+$ cells were analyzed for Foxp3 expression. Shown is the mean percentage ± SEM of Foxp3 expression in transferred cells. Similar results were also obtained in separate experiments in which IFN-γ was neutralized by Ab injection.

and mature cells from NG-BAC IFN-γ$^{-/-}$ mice with TGF-β and at-RA resulted in equal or higher Foxp3 expression in mature cells (Fig. 7D), but RTEs continued to show increased sensitivity to at-RA with regard to CD103 (RTE 65% CD103$^+$ versus mature 19% CD103$^+$ at 0.01 nM at-RA; $p < 0.05$) (Fig. 7D, 7E) and CCR9 expression (Fig. 7E). We further predicted that if the silencing of IFN-γ through enhanced retinoic acid signaling was important in the upregulation of Foxp3 in RTEs, inhibition of retinoic acid signaling would eliminate the differences in Foxp3 expression and restore IFN-γ secretion in RTEs. To test this possibility, sorted RTEs and mature naive cells were stimulated in the presence of TGF-β, low dose at-RA, and 1 μM LE540, an inhibitor of retinoic acid signaling (42). As predicted, LE540 treatment of RTEs reduced the percentage of Foxp3$^+$ cells that developed (4.4 ± 0.9%) and increased IFN-γ expression (Fig. 7F). When retinoic acid signaling was inhibited with LE540, the addition of anti–IFN-γ partially restored Foxp3 expression in RTEs (8.2 ± 0.9%, $p < 0.05$). This confirms that in the setting of impaired retinoic acid signaling, the decreased expression of Foxp3 in RTEs is at least partially mediated by increased IFN-γ production. Thus, in RTEs, enhanced RAR signaling contributes to decreased IFN-γ production, which in turn permits the upregulation of Foxp3. In mature naive cells, decreased RAR signaling contributes to increased IFN-γ production, which in turn silences Foxp3 expression.

Discussion

In a series of seminal experiments, Billingham et al. (43) described a process of actively acquired tolerance in which fetal animals exposed to peripheral foreign Ags were rendered tolerant to future exposures of the same Ag. From this work emerged the concept of neonatal tolerance whereby "antigens which impinged upon an animal sufficiently early in its life should come to be accepted as if they were its own" (44). In recent years, the view that the neonatal period represents a window for establishing tolerance has faded from prominence as the mechanisms of central thymic tolerance have been recognized and investigated. Nevertheless, it remains true that the neonatal period is a unique window of time in which peripheral T cell tolerance is more easily established. This is demonstrated clinically by the reduction in the likelihood of the development of graft-versus-host disease in the recipients of mismatched allogeneic umbilical cord blood transplants carrying neonatal T cells compared with recipients of bone marrow transplants carrying adult T cells (45).

Our results suggest that another window of time for peripheral tolerance exists at the cellular level with newly minted recent thymic emigrant T cells maintaining a tolerance-prone state for a period in the periphery before they fully mature. We demonstrate that across a wide range of conditions in vitro and in vivo, CD4$^+$ RTEs are more permissive to Foxp3 upregulation and tolerance induction than are their mature naive CD4$^+$ T cell counterparts. The increased Foxp3 induction in RTEs is mediated at the molecular level by two interlinked mechanisms: increased sensitivity to retinoic acid and decreased production of the proinflammatory cytokine IFN-γ. In CD4$^+$ RTEs, the increased expression of RARs results in the induction of the retinoic acid–responsive genes Foxp3, CD103, and CCR9 at much lower concentrations of at-RA

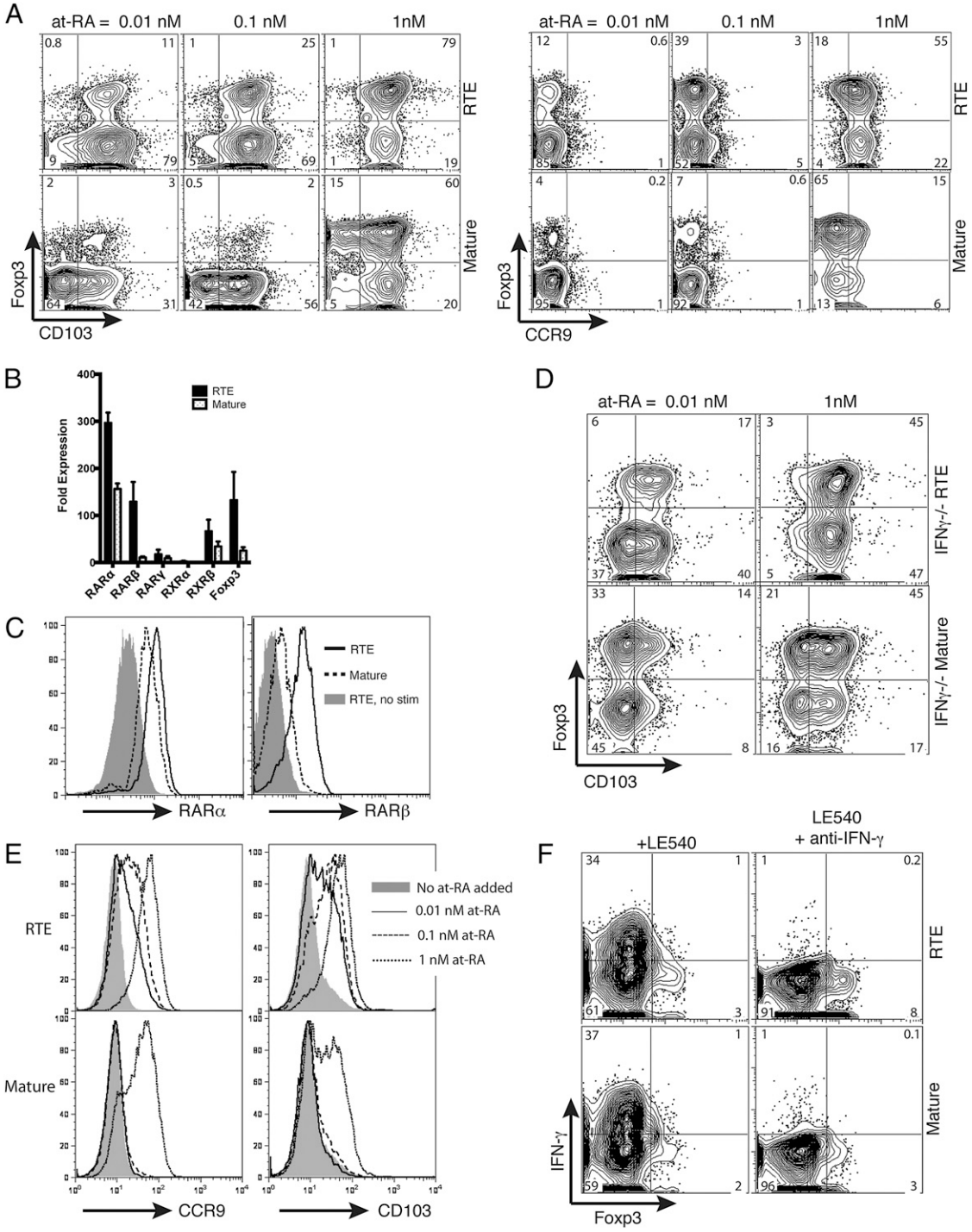

FIGURE 7. RTEs display increased sensitivity to at-RA. (**A**) Increased at-RA–dependent CD103 and CCR9 induction in RTEs. Sorted GFP$^+$ RTEs and GFP$^-$ mature naive CD4$^+$ T cells from NG-BAC Rag$^{-/-}$ DO$^+$ mice were stimulated with anti-CD3/anti-CD28 microbeads, 5 ng/ml TGF-β, and at-RA as indicated. On day 5 of culture, the cells were collected and stained for Foxp3 and CD103 (*left panels*) or CCR9 (*right panels*). Data are representative of three separate experiments. (**B**) Real-time PCR analysis of retinoic acid receptor expression in RTEs. Total RNA was collected from GFP$^+$ RTE and GFP$^-$ mature naive CD4$^+$ T cells that had been stimulated with anti-CD3/anti-CD28 microbeads for 48 h in the presence of TGF-β. Shown are the mean fold differences ± SD in expression of each gene relative to expression of RXRα in mature cells (after first normalizing samples by 18S gene expression) from three separate experiments. (**C**) Intracellular staining for RARs. Sorted RTE and mature cells were stimulated for 48 h with TGF-β (5 ng/ml) and retinoic acid (0.1 nM), stained for intracellular RARα (*left*) or RARβ (*right*), and analyzed by flow cytometry. RTE cells prior to stimulation are shown as controls. Similar results were obtained in two separate experiments. (**D** and **E**) Increased responsiveness of RTEs to retinoic acid is independent of IFN-γ. RTE and mature cells were sorted from NGBAC IFN-γ$^{-/-}$ mice and stimulated as in (A). Shown in (D) are representative FACS plots of at-RA–mediated CD103 induction. Shown in (E) are histogram plots of CD103 and CCR9 induction across a range of at-RA concentrations. Mature cells stimulated without at-RA addition are included on each plot for comparison (gray histograms). Similar results were obtained in two separate experiments. (**F**) Inhibition of retinoic acid signaling restores IFN-γ secretion and inhibits Foxp3 expression in RTEs. Sorted GFP$^+$ RTEs and GFP$^-$ mature naive cells were stimulated with anti-CD3/anti-CD28 microbeads in the presence of 5 ng/ml TGF-β and the retinoic acid antagonist LE540 (1 μM) with or without anti–IFN-γ (10 μg/ml) as indicated. On day 5 of culture, the cells were stained for intracellular IFN-γ and Foxp3. Similar results were obtained in three separate experiments.

than those needed to induce them in mature naive $CD4^+$ cells (Fig. 7). Owing in part to this increased sensitivity to at-RA, RTEs also make less IFN-γ (Figs. 3) and thus are not subjected to the autocrine IFN-γ–mediated repression of Foxp3 observed in mature naive cells (Figs. 4, 6). Even the low level of retinoic acid present at baseline in media is sufficient to suppress IFN-γ production in RTEs, and the inhibition of at-RA signaling with the retinoic acid antagonist LE540 partially restores IFN-γ production and reduces Foxp3 induction in RTEs (Fig. 7F). In contrast, mature naive $CD4^+$ T cells are much less sensitive to at-RA and produce much more IFN-γ, which leads to the silencing of Foxp3 expression in an autocrine or paracrine fashion (Figs. 4–6). This inhibition of Foxp3 induction in mature naive cells can be overcome through the blockade of IFN-γ (Figs. 6B, 7A) or by high doses of at-RA (Fig. 2C).

These mechanisms for the induction of peripheral tolerance in RTEs seem particularly suited to facilitate tolerance to gut-derived exogenous Ags. The large burden of Ags delivered to the gut constitutes the biggest potential "hole" in thymic tolerance that must be dealt with to maintain immune homeostasis. The increased sensitivity of RTEs to retinoic acid would provide a convenient mechanism to facilitate the induction of peripheral tolerance to these Ags, as gut APCs are known to produce retinoic acid (16, 19, 22, 34, 35). Consistent with this idea, we found that the percentage of cells that converted to the Foxp3 phenotype in the transferred polyclonal RTE populations was highest in the gut (Fig. 1B). Interestingly, $CD8^+$ RTEs have been reported to home directly to the mucosa of the small intestine (46). In preliminary experiments, we have not observed homing of $CD4^+$ RTEs directly to the lamina propria, but migration to the MLN is easily detected. It is also unclear whether similar mechanisms maintain tolerance to self-Ags in tissues where retinoic acid may not be as abundant, and we are currently addressing this question.

Our results suggest that $CD4^+$ RTEs are actively being converted into $Foxp3^+$ cells in the periphery in our adoptive transfer experiments (Figs. 1, 3, 6C). Possible alternative interpretations might be that the percentage of $Foxp3^+$ cells in the recovered RTEs increases because of poor survival of the $Foxp3^-$ RTEs or that there is preferential expansion of the small number of pre-existing $Foxp3^+$ cells in transferred RTE cells. Decreased survival of RTEs relative to mature naive cells after adoptive transfer has been reported by others (47). Although all of these mechanisms are possible, we think that active conversion is the dominant mechanism at play in our experiments for three reasons. First, we do not see consistent differences in total cells recovered in our experiments. In a direct comparison of in vivo survival of allelically marked RTE and mature cells during oral tolerance, total survival was equivalent, but increased numbers of Foxp3 cells were seen in the RTE population (Fig. 3C, 3D). Second, the increase in the percentage of $Foxp3^+$ cells was most pronounced in the GALT, consistent with an at-RA–driven conversion. Third, the initial percentage of $Foxp3^+$ cells in the NG-BAC $Rag2^{-/-}$ DO11.10 cells is too low to make the expansion of these cells plausible as the dominant mechanism in the oral tolerance and in vitro experiments. Nevertheless, we cannot rule out the possibility that some deletion of RTEs is also occurring. We also think that although IFN-γ is important in this process, other proinflammatory cytokines are likely to play a role as well. For example, we have observed that mature naive $CD4^+$ cells produce significantly higher levels of IL-21 after stimulation in vitro under Treg-inducing conditions than RTEs. The blockade of IL-21 also increases Foxp3 induction in mature naive $CD4^+$ T cells (data not shown), but the relationship between IL-21 and IFN-γ and the relative importance of each in this system are still under investigation.

Where overlap exists, our results are largely in agreement with other published results. Similar to our findings in the murine system, Haines et al. (25) demonstrated that human $CD4^+$ RTEs produce less IL-2 and IFN-γ after stimulation in vitro. In the murine system, two other groups have found decreased IFN-γ production in RTEs compared with mature cells (26, 27). In the same study, however, Hendricks and Fink (26) also reported increased Treg lineage commitment in mature cells relative to RTEs, but the mechanism by which mature cells were able to achieve increased Foxp3 expression in the face of higher local IFN-γ concentrations was not addressed. It is possible that the low and variable levels of anti-CD3 (30–500 ng/ml) used for stimulation or other subtle differences in culture conditions might have resulted their findings.

In summary, we have shown that RTEs display a phenotype of increased sensitivity to retinoic acid and decreased inflammatory cytokine production that promotes Foxp3 induction and peripheral tolerance. The maintenance of tolerance to self-Ags and benign environmental Ags is vital as evidenced by the dramatic autoimmune phenotype that results when Foxp3 is mutated or Tregs are ablated. Delayed maturation of RTEs may provide redundancy in the system to help protect the host against inappropriate inflammatory immune responses. These mechanisms likely contribute to the ease with which tolerance is achieved in neonates, a phenomenon first described by Medawar and colleagues (43), as RTEs comprise the bulk of the neonatal peripheral T cell compartment. In settings such as viral infections in adults where mature naive T cells or memory cells provide abundant IFN-γ, the induction of tolerance in RTEs may be overridden by IFN-γ signaling. Whereas this may allow RTEs to participate in immune responses against pathogens in adults, it also provides a potential mechanism for the induction of autoimmunity, a phenomenon relatively common in adults but exceedingly rare in neonates. These mechanisms may also help to explain the relatively weak immune responses observed after bone marrow transplant, a time in which RTEs are abundant in the peripheral T cell pool.

Acknowledgments

We thank Pamela Fink for providing C57BL/6J background NG-BAC mice.

Disclosures

The authors have no financial conflicts of interest.

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