Retinoic Acid Enhances Foxp3 Induction Indirectly by Relieving Inhibition from CD4+CD44hi Cells

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SUMMARY

CD4+Foxp3+ regulatory T (Treg) cells originate primarily from thymic differentiation, but conversion of mature T lymphocytes to Foxp3 positivity can be elicited by several means, including in vitro activation in the presence of TGF-β. Retinoic acid (RA) increases TGF-β-induced expression of Foxp3, through unknown molecular mechanisms. We showed here that, rather than enhancing TGF-β signaling directly in naïve CD4+ T cells, RA negatively regulated an accompanying population of CD4+ T cells with a CD44hi memory and effector phenotype. These memory cells actively inhibited the TGF-β-induced conversion of naïve CD4+ T cells through the synthesis of a set of cytokines (IL-4, IL-21, IFN-γ) whose expression was coordinately curtailed by RA. This indirect effect was evident in vivo and required the expression of the RA receptor alpha. Thus, cytokine-producing CD44hi cells actively restrain TGF-β-mediated Foxp3 expression in naïve T cells, and this balance can be shifted or fine-tuned by RA.

INTRODUCTION

Foxp3+CD4+ regulatory T (Treg) cells are central to the maintenance of immunological homeostasis and tolerance in the T lymphocyte compartment (Sakaguchi et al., 2006). This role is exemplified by the devastating lymphoproliferation and multi-organ autoimmunity that occur in mice or humans deficient in this population, whether through the carrying of spontaneous mutations (scurfy mice, IPEX patients) (Ziegler, 2006) or through experimental lineage ablation (Kim et al., 2007). A distinct gene-expression signature characterizes Foxp3+ Treg cells (Fontenot et al., 2005; Huehn et al., 2004; Herman et al., 2004; Hill et al., 2007). Foxp3 plays an important role in determining this signature but is not the master regulator it was once thought to be, because it is neither sufficient to elicit the full Treg cell genomic profile nor strictly necessary for generation of the lineage (Gavin et al., 2007; Lin et al., 2007; Hill et al., 2007).

Most of the Treg cells present in lymphoid organs of normal mice are generated in the thymus (Hsieh et al., 2006; Pacholczyk et al., 2006; Wong et al., 2007a), and the specific TCR repertoire that distinguishes them from conventional CD4+ T cells (Tconv) can be tracked from the thymus to peripheral lymphoid organs (Hsieh et al., 2006; Pacholczyk et al., 2006; Wong et al., 2007b). In addition, mature CD4+ T cells from peripheral lymphoid organs can be converted to Foxp3 positivity in a variety of conditions: in chronic suboptimal stimulation by agonist peptide (Kretschmer et al., 2005; Apostolou and von Boehmer, 2004), after exposure to orally administered agonist (Mucida et al., 2005; Coombes et al., 2007), or during lymphopenia-driven homeostatic expansion (Sun et al., 2007). Finally, activation in the presence of the cytokines IL-2 and TGF-β in vitro can induce Foxp3 expression in naïve Tconv cells, which then acquire some characteristics of Treg cells, including suppressive properties in some contexts (Chen et al., 2003; Fantini et al., 2004; Wan and Flavell, 2005). In contrast, Foxp3 expression in TGF-β-induced Treg cells is unstable (Floess et al., 2007); these cells are not suppressive in all assays, and converted cells acquire only a partial segment of the genomic signature typical of Treg cells (Hill et al., 2007).

Most interesting in this context were the observations that dendritic cells (DCs) from gut origin, in particular a CD103+ population from the lamina propria (LP), can markedly enhance TGF-β-induced conversion of CD4+ T cells to the Foxp3+ phenotype in vitro and that this effect can be ascribed to all-trans retinoic acid (RA), which also represses differentiation to an IL-17-secreting phenotype (Mucida et al., 2007; Sun et al., 2007; Coombes et al., 2007; Benson et al., 2007; Elias et al., 2008). RA, the key metabolite of Vitamin A, is an important morphogen that affects the development and maintenance of a wide variety of tissues, as exemplified by the pleiotropic abnormalities that appear in Vitamin-A-deficient embryos or adults (reviewed in Mark et al., 2006). Concerning hematopoietic cells, RA can have general stimulatory effects on lymphocyte responses, possibly by inhibiting apoptotic pathways (Iwata et al., 2004), and affects natural killer (NK) cell activity by modulating interferons and NK cell ligands (Abb et al., 1982a; Abb et al., 1982b; Cerwenka
Counterconversion and Retinoic Acid

et al., 2000). In addition, RA seems to play a predominant role in the homeostasis and homing of lymphoid populations of the gut-associated lymphoid tissue (GALT). It is synthesized in abundance by gut DCs (Iwata et al., 2004; Coombes et al., 2007), induces the specific gut-homing molecules CCR9 and α4β7 integrin on T cells, and also promotes GALT-related functions in B cells (Iwata et al., 2004; Mora et al., 2006). RA’s important role in controlling Foxp3 expression mediated by TGF-β also suggests that the GALT has evolved a specific system to maintain a balanced symbiosis between the gut flora and the immune system (Iwata et al., 2004; Mora et al., 2006; Mucida et al., 2007; Sun et al., 2007; Coombes et al., 2007; von Boehmer, 2007).

RA receptors (RARs) belong to the family of nuclear hormone receptors and act as ligand-dependent transcriptional regulators. There are three subtypes of RARs (RARα, RARβ, and RARγ) (Chambon, 1994), all of which bind all-trans RA at high affinity, but each has distinct developmental effects and genomic footprints. RARα and RARγ are the predominant forms expressed in immunological cells (Purton et al., 2006). More recently, it has been realized that RA can also serve as an activating ligand for the PPARα and PPARβ receptors, with different effects on cell growth and apoptosis (Schug et al., 2007).

In this context, it was clearly of interest to investigate the molecular mechanisms by which RA promotes Foxp3 expression mediated by TGF-β. Beyond their direct effects as transactivators, RARs also influence transcription by transrepression of AP-1 activity (Nicholson et al., 1990; Salbert et al., 1993; Chen et al., 1995; Altucci and Gronemeyer, 2001). This could conceivably affect Foxp3 expression in many ways: by altering signals induced by T cell costimulation (Wu et al., 2006) or by disturbing the competition between AP-1 and Foxp3 for NFAT binding (von Boehmer, 2007) and, therefore, any downstream events. Alternatively, liganded RARs could potentiate TGF-β signaling. The explorations reported here started from these premises but ended up with a rather different conclusion, unveiling a multiscell interplay underlying RA’s action.

RESULTS

Treg Cell Conversion and Homeostasis in RAR-Deficient Mice

Three main nuclear receptors for RA have been described. Recent studies, using pharmacological inhibitors, have implicated RARα in RA-boosted Foxp3 induction (Schambach et al., 2007; Kang et al., 2007; Elias et al., 2008), but we felt it worthwhile to exploit genetically deficient mice to evaluate the individual contributions of the three receptors, as well as to assess the impact of the RA pathway on Treg cell populations in vivo. Thus, mice with loss-of-function mutations in Rara, Rarb, or Rarg (Chapellier et al., 2002a; Chapellier et al., 2002b) were bred and analyzed. Purified CD4+ Foxp3+ T cells from each of the mutant lines (or their control littermates) were stimulated in vitro by CD3 monoclonal antibodies together with WT splenic DCs and TGF-β, with or without RA. As expected, the proportion of Foxp3+ cells was boosted by RA in cultures from control littermates and from Rarb-deficient and Rarg-deficient mice, but the effect was completely abrogated by the Rara-deficient mutation (Figures 1A and 1B). Similarly, the induction of α4β7 integrin by RA was dependent on RARα (Figure 1C).

To investigate the impact of this blockade of RA signaling on the selection and steady-state frequency of Treg cell populations in vivo, we analyzed CD4+ lymphocytes from the thymus, secondary lymphoid organs, and GALT for expression of Foxp3 and CD25 (Figure 1D). Positive selection of Treg cells in the thymus appeared unaffected, with the usual proportion of Foxp3+ cells among CD4+CD3+ thymocytes. Steady-state frequencies of Treg cells were not decreased in the lymphoid organs of Rara–/– mice; if anything, they showed an increase in the LP. Thus, a deficiency in signaling through the RARα did not decrease the overall frequency of Treg cells, whose steady-state frequency appears to be set by other factors. Correspondingly, Rara–/– mice do not present with obvious autoimmune manifestations.

Impact of RA on the Treg Cell Signature

As reported previously, Foxp3+ cells induced by TGF-β in the presence of IL-2 and TCR activation cannot be equated to bona fide Treg cells, because they are missing important elements of the Treg cell gene-expression signature (Hill et al., 2007); TGF-β-induced Foxp3 expression is unstable and can be rapidly lost upon removal of the cytokine, consistent with a different chromatin state at the Foxp3 locus (Floess et al., 2007). One possible explanation for the RARα-mediated effect was that RA released converted cells to acquire the “missing segment” of the Treg cell signature, thus resulting in a more complete and stable phenotype (in preliminary experiments, cells converted in the presence of LP DCs did appear to better maintain their Foxp3+ phenotype upon in vivo transfer than did those generated in the presence of spleen [Sp] DCs; see also Benson et al., 2007). Thus, we compared the gene-expression profiles of Foxp3+ cells, generated by 5 days of TGF-β treatment from sorted CD4+ Foxp3+ cells, in the presence of LP or Sp DCs, the latter with or without 10 nM RA. The gene-expression profiles were obtained from purified T cell populations, with Foxp3-eGFP reporter mice used as donors. We focused particularly on the transcripts that constitute the robust “Treg cell signature,” derived from multiple data sets in a previous study (Hill et al., 2007).

In the graphs of Figure 2A, the expression profiles of Foxp3+ cells generated with TGF-β and LP DCs versus RA supplementation are compared in parallel to those from Foxp3+ cells generated simply with TGF-β and splenic DCs, a condition that leads to less conversion (Sun et al., 2007). Although there was no general displacement, a subtle but distinct off-diagonal shift of the Treg cell signature transcripts was observed, those normally overrepresented in Treg cells being, as a group, slightly more expressed in samples from the LP DC cocultures (red dots, 271 versus 125, p = 1.2 × 10−11). Accordingly, transcripts underrepresented in Treg cells were also underexpressed in Foxp3+ cells from LP DC cocultures (blue dots, 125 versus 71, χ2 p = 1.2 × 10−6). These biases were also observed with Treg cell signature transcripts in Foxp3+ cells from RA-supplemented cultures (219 versus 188, 52 versus 144, χ2 p = 4.9 × 10−11). A direct comparison of transcripts affected by RA or LP DCs showed a clear parallel, supporting the notion that the ability of LP DCs to enhance Foxp3 expression is due to the production of RA (Figure S1, available online). Thus, the presence of RA did appear to slightly reinforce the Treg cell gene-expression signature. But did it promote complementation of the “holes,” i.e., those transcripts normally
RARα Is Responsible for the Enhanced Conversion Mediated by Retinoic Acid

(A) CD4^+CD25^− T cells were sorted and cultured in vitro with WT CD11c^+ DCs (ratio 10:1, T cell to DC) in the presence of anti-CD3 (1 μg/ml) and TGF-β (10 ng/ml), with or without 10 nM RA, for 5 days. Foxp3 and α4β7 expression was determined in CD4^+ T cells from Rara^−/− mice and compared to WT littermate controls (representative FACS plot from two or more independent experiments).

(B) Summary of TGF-β-mediated conversion in cultures from WT or Rara^−/−, Rabr^−/−, or Rarg^−/− mice treated with or without RA, as described in (A).

(C) The effects of RARα on α4β7 expression in CD4^+ T cells after treatment with TGF-β in the presence (open histogram) or absence (filled histogram) of RA, as described in (A). Representative FACS plot from two or more independent experiments is shown.

(D) The proportion of CD4^+Foxp3^+ T cells in the lamina propria, thymus, or spleen from WT and Rara^−/− mice was compared. Organs were processed from WT or Rara^−/− mice, and Foxp3 and CD25 expression on CD4^+ T cells was determined by FACS. Numbers in the gates represent the mean (± SD where applicable) for Foxp3 expression (thymus, n = 2; spleen, n = 5; lamina propria, n = 8), and the p value was determined by Student’s t test.
overexpressed in Treg cells that are not induced in TGF-β-elicited Foxp3+ cells (Hill et al., 2007)? This question was addressed by the signatureMatch analysis of Figure 2B. This algorithm is designed to test how well a signature is achieved in test populations. It uses normalized expression values that are then standardized relative to two reference populations that define the expression minima and maxima for each transcript of the signature (here ex vivo Treg and Foxp3− Tconv cells, pops. 1 and 2). In agreement with our previous report, in vitro activation in the presence of IL-2 (pop. 3) induced a substantial fraction of the Treg cell signature, and the addition of TGF-β (pop. 4) brought forth only a minor subset of Treg cell signature transcripts (mainly in the TGF bracket). Although there was an additional impact of LP DCs in the cultures (pop. 5), partially mimicked by Sp DCs + RA (pop. 7), much of the Treg cell signature transcripts remained at the basal level of Tconv cells. Thus, the “holes” in the signature elicited by activation in the presence of TGF-β largely persist in spite of RA. The FoldChange plots of Figure S2 confirmed this impression, and there were substantial changes in only a small number of Treg cell signature genes. This inability of RA to complement the partial effect of TGF-β on the Treg cell signature is also evident from the representation of Figure 2C, which compares the effect of RA (in the presence of TGF-β) to the Treg cell profile: only a few genes of the Treg cell signature were directly affected in any appreciable manner by RA (e.g., Nrp1, P2rx7).

Most of the changes elicited by RA were independent of the Treg cell signature, a conclusion bolstered by the plot of Figure 2D, which compares the effects of RA in Foxp3+ cells versus in cells remaining Foxp3− in those cultures: RA-responsive transcripts were very similar in both cell types. The RA signature (listed in Tables S1 and S2) included some of the transcripts one expects to be induced, such as Ccr9 and Itga4 (x4 integrin), but also some repressed transcripts. It did not show any substantial overlap with the “TGF-β signature,” i.e., the transcripts affected by TGF-β treatment independently of Treg cell conversion (Hill et al., 2007), indicating that RA does not enhance conversion by increasing the intensity of TGF-β signaling. In contrast, the observation that the IL-6 receptor was downregulated by 75 percent was quite suggestive because IL-6, in the presence of TGF-β, promotes the expression of IL-17, while inhibiting that of Foxp3 (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006; Stockinger and Veldhoen, 2007).

**IL-6 Is Only a Minor Player in RA’s Action**

The repression of IL-6Rα by RA observed in the gene-expression profiles suggested a mechanism for RA’s action: because IL-6 inhibits the induction of Foxp3, RA might simply be reducing the sensitivity of naive T cells to inhibition by IL-6. We first confirmed by flow cytometry that the alpha chain of IL-6R was indeed repressed by RA (Figure 3A; over six experiments, the mean fluorescence intensity dropped from 526 ± 116 to 255 ± 81, p < 0.001). This regulation of IL-6Rα expression was also lost when cells from Rara−/− mice were tested (Figure 3B). Under our culture conditions, addition of recombinant IL-6 did have the expected inhibitory effect on Foxp3 expression, and this influence was partially reversed by RA, consistent with the notion that RA might help to relieve the inhibition by IL-6 (Figure 3C). In contrast, RA was still very effective in cultures of cells from IL-6-deficient mice; if anything, its influence was even stronger than that in cultures from WT mice (Figure 3D). Thus, an inhibition of IL-6 action, although it may partially contribute to RA’s impact on Foxp3 expression, particularly in IL-6-rich environments, cannot be the main mechanism through which RA promotes TGF-β-mediated Foxp3 expression.

**RA Acts Indirectly on Treg Cell Conversion**

The rather limited impact of RA on transcriptional profiles of converted Treg cells raised the possibility of an indirect effect; of RA acting on something other than the responding cells. This hypothesis emerged to the forefront by a serendipitous observation. In experiments designed to test the effect of RA on isolated CD4+ T cell subsets, we observed that the enhancing effect of RA was lost when the fully naive fraction of CD4+ cells (purified as CD44+/CD62Lhi) were used as responders (Figure 4A), rather than CD4+ T cells from unfractionated splenocytes (Figure 4B). Also apparent in these experiments was that purified naive CD4+ cells gave rise to a higher proportion of Foxp3+ convertants when cultured alone than when cultured as part of unfractionated splenocytes (compare the left panels of Figures 4A and 4B). The insensitivity of naive CD4+ cells to RA was not due to a saturating response to TGF-β, given that it was also observed through suboptimal doses of TGF-β (Figure 4C). Similarly, the conversion of purified naive cells could not be boosted through a range of RA doses (Figure 4D). This lack of response did not mean that purified naive CD4+ T cells were refractory to RA, given that CCR9 and the α4β7 integrin were induced effectively in these cells by RA exposure (not shown).

These data suggested that the increased generation of Foxp3+ cells in response to RA in these cultures of unseparated splenocytes might represent the lifting of an inhibition imparted by other cells, a dampening lifted equally by RA or by purification of the naive responder cells. It seemed likely that the inhibitory cell might also be a CD4+ T cell, given that it was clearly possible to obtain strong responses to RA when whole CD4+ populations were used as responders to TGF-β (as in the experiments of Figures 1 and 2, and as in all of the initial descriptions of RA’s effect [Mucida et al., 2007; Lufkin et al., 1993; Coombs et al., 2007; Benson et al., 2007; Elias et al., 2008]). This inhibitory population would then be predicted to have a CD44hi memory phenotype and would thus be lost during the purification of naive responder cells. We directly tested this hypothesis by the reconstitution experiments depicted in Figure 5, in which CD4+ cells were purified as CD44-negative or CD44-positive (each identifiable through CD45 allotypes so as to track their fate after culture) and admixed in stimulation cultures supplemented or not with RA. The induction of Foxp3 in naive cells was clearly inhibited by coculture with an equal number of CD44hi cells, an inhibition reversed by the addition of RA (Figure 5A; several experiments tabulated in Figure 5B). This inhibitory effect of CD44hi cells (hereafter referred to as “counterconversion” for brevity), as well as its reversal by RA, could be titrated through a range of cell ratios (Figure 5C). This dose-response range was compatible with the results obtained with whole CD4+ cells (e.g., Figure 4), in which the CD44+/CD44− ratio was approximately 4.

We then asked how CD44hi memory T cells influence the TGF-β-induced conversion of naive T cells, the production of a soluble factor being the most likely candidate. Culture supernatants from...
Figure 2. Retinoic Acid Influences the Expression of a Discrete Group of Genes that Are Largely Independent of the Canonical Treg Cell Genomic Signature

(A) Comparison of probe-expression values in Foxp3+ T cells after culture with anti-CD3, TGF-β, and: lamina propria DCs (y axis) or spleen DCs (x axis), left panel; or spleen DCs with (y axis) or without (x axis) RA (100 nM), right panel. Probes highlighted in red and blue correspond to genes either upregulated or downregulated (respectively) in the canonical Treg cell genomic signature.

(B) signatureMatch heat-map analysis of the Treg cell genomic signature in ex vivo Treg cells or TGF-β-converted cells (groups 3 and 4 are from Hill et al., 2007). Raw expression values were normalized to 1 or zero for ex vivo Treg cells or Tconv cells, respectively. The expression values for genes from the TGF-β-converted cells were normalized within this range (for a detailed description of the algorithm, see Supplemental Experimental Procedures) and displayed as a heat map, in which red represents the expression of a gene at the same or a greater level than what is found in an ex vivo Treg cell, whereas black represents the expression of...
the inhibitory factor(s) required stimulation of the CD44hi population, rather than through the naive converting cells themselves, we exploited the identification of RARα, the primary receptor in this context, and conducted mixed cell experiments using naive or memory populations from Rara−/− mice or congenically marked wild-type (WT) mice (Figure 5G). Under these conditions, expression of Foxp3 in naive cells from Rara−/− mice could still be increased by RA. In contrast, RA was without effect when memory cells from Rara−/− mice were cocultured with WT naive cells. As expected, the genotype of the naive T cells was immaterial (Figure 5G). Thus, the primary mode of action of RA in enhancing the conversion to a Foxp3+ phenotype appears to be through the reduction of inhibitory factors produced by CD44hi cells.

Counterconversion Results from Synergistic Cytokine Action

We then sought to identify the soluble factor(s) mediating counterconversion. Such a factor should have two characteristics: be produced by CD44hi CD4+ cells and be reduced by RA treatment. Triplicate microarray profiles were generated on CD45-alloype-marked CD44hi cells in stimulated cocultures prepared exactly as in Figure 5, and these were compared with or without RA treatment. The results are displayed on the Expression versus FoldChange plot of Figure 6A, in which Gene Ontology identifiers were used for identifying cytokines and proteins released in the extracellular space (shown as red dots). Several factors immediately stood out in this analysis, matching criteria of both transcript abundance and repression by RA: IL-4, IL-9, IL-10, IL-21, and Interferon-γ (IFN-γ). This analysis also confirmed the RA responsiveness of these cells, given that RA-responsive transcripts identified in Figure 2 were also found differentially expressed in CD44hi cells (i.e., Ifng4 and Ccr9). In contrast, the vast majority of secreted proteins were unaffected by RA or were even induced (e.g., granulin). Counterconversion was not likely to be mediated a gene that is at the same or a lower level than that found in an ex vivo Tconv cell. Numbers to the right of the diagram show the average score for each group across the entire signature.

(C) FcFc plot comparing the effects of RA in Foxp3+ TGF-β-converted cells (y axis) with ex vivo Treg cells (x axis).

(D) FcFc plot comparing the effects of RA in Foxp3+ (y axis) and Foxp3− (x axis) TGF-β-treated cells.
none of these cytokines were detected in naive CD44

and IL-10 had no effect. IL-21 was inhibitory as expected, but

only at relatively high doses. IFN-γ had a modest effect, but

IL-4 led to a marked decrease of Foxp3 expression, consistent

with recent studies (Wei et al., 2007; Mantel et al., 2007). Anti-

body-blockade experiments were performed for determining

which of these cytokines played the most important part in coun-

terconversion in coculture experiments (Figure 6C). Blocking

IL-21 or IFN-γ individually had little or no effect, but anti-IL-4

antibodies had a significant impact on counterconversion activ-

ity (the importance of IL-4 was also apparent from the use of

CD44hi cells from Il4−/− mice; Figure S4). Pairwise combinations

of antibodies had little more effect than did each alone, but

blockade of all three cytokines was more effective at eliminating

counterconversion activity, albeit still incompletely (Figure 6C). It

should also be noted that these blocking antibodies did not

affect Foxp3 expression in cultures with naive T cells only, paral-

leling the insensitivity to RA that was seen (data not shown).

This result prompted us to further explore whether these cyto-

kines act synergistically to inhibit Foxp3 expression by adding

them back to conversion cultures of purified naive T cells at suboptimal doses (Figure 6D). At the doses used, IFN-γ and

IL-21 had little effect on their own and IL-4 lead to only a partial

reduction in Foxp3 expression. Limited synergy was seen when

cytokines were added in pairs; for example, IL-21 enhanced the

inhibitory effects of IFN-γ and IL-4. The largest reduction of

Foxp3 expression was seen when all three cytokines were added

at once, confirming their three-way synergy.

Thus, CD44hi memory T cells release a set of cytokines that

inhibit TGF-β-driven Foxp3 expression. Their effect seems to be

elicited by their combined and synergistic action, rather than by

any of them alone, and RA inhibits the transcription of the whole

panel.

Figure 4. Retinoic Acid Acts Indirectly on Foxp3 Expression

(A) RA does not enhance TGF-β-mediated Foxp3 expression in purified naive

CD4+ T cells. Naive CD25−CD44hiCD62Lhi CD4+ T cells (0.5 × 10⁶) were acti-

vated with anti-CD3 and anti-CD28 beads and TGF-β (10 ng/ml) in the absence

(left panel) or presence (middle panel) of RA (100 nM) for 4 days. Representative

FACS data are shown for Foxp3 expression with the results of multiple experi-

ments plotted in the right panel (n = 6, p value for Student’s t test).

(B) RA does enhance TGF-β-mediated Foxp3 expression in CD4+ T cells from

unfractionated splenocytes. Splenocytes (0.5 × 10⁶) were activated in vitro (as

in [A]) in the absence (left panel) or presence (middle panel) of RA (100 nM) for

4 days. Representative FACS data are shown for Foxp3 expression in CD4+

T cells with the results of multiple experiments plotted in the right panel

(n = 6, p value for Student’s t test).

(C) RA does not enhance Foxp3 expression in purified naive CD4+ T cells over a

range of TGF-β concentrations. Naive CD4+ T cells (purified as in [A]) were

activated with anti-CD3 and anti-CD28 beads in the presence of 100 nM RA

and the indicated doses of TGF-β; then analyzed at day 4 for Foxp3 expression

by FACS. Representative data are shown for three independent experiments.

(D) Foxp3 expression is not enhanced in naive CD4+ T cells over a range of RA

doses. Naive CD4+ T cells were activated as in [A] in the presence of the

indicated concentrations of RA, then analyzed for Foxp3 expression by

FACS. Summarized data from three independent experiments is shown.

by IL-6, because the CD44hiCD4+ cells produced virtually no IL-6

transcript.

In order to confirm the regulation of expression of some of these cytokines by RA, we performed intracellular staining of the mem-

ory cells (identified in the cocultures as CD45.1 allotype and devoid of

memory cells (identified in the cocultures as CD45.2)) after activation

for 48 hr. Clear reductions in IL-10 and IL-4 production were

evident in memory T cells treated with RA (Figure S3). Somewhat

surprisingly, we did not see the same trend for IFN-γ; possibly
due to a more complex posttranscriptional regulation. Notably,

none of these cytokines were detected in naive CD44hi T cells

cultured under the same conditions (not shown).

We then tested the effect of these candidate cytokines on

TGF-β-induced conversion of naive CD4+ cells (Figure 6B). IL-9

and IL-10 had no effect. IL-21 was inhibitory as expected, but

only at relatively high doses. IFN-γ had a modest effect, but

IL-4 led to a marked decrease of Foxp3 expression, consistent

with recent studies (Wei et al., 2007; Mantel et al., 2007). Anti-

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RA Acts Indirectly on Antigen-Specific Conversion in GALT

To verify the in vivo relevance of the indirect mode of RA action demonstrated in vitro, we exploited the known role of RARα in the process, asking how the same WT CD4+ T cells would convert when transferred into WT or Rara−/− hosts. We used as a model Foxp3-negative OT-II T cells transferred into hosts also

fed with oral ovalbumin. This model of in vivo antigen-specific conversion is restricted to the GALT, because conversion does

not occur in subcutaneous lymph nodes or in spleen (Sun

et al., 2007). Consistent with previous findings, transferring OT-II

Rag1−/− T cells (identifiable with a CD45.1 allotype and devoid of

any detectable Foxp3+ cells) to either WT or Rara−/− hosts led to
detectable conversion in both the lamina propria and the mesen-
	heric lymph nodes of ova-fed mice (Figure 7A; as expected, no

conversion was observed in the spleen or other lymph nodes); OT-II cells in WT recipients showed a 2-fold higher proportion

of Foxp3+ cells compared to those in Rara−/− hosts (Figure 7B). These data provide direct evidence that RA signaling via RARα affects Foxp3 induction in vivo in an indirect manner, through cells other than the naive CD4+ cells.

DISCUSSION

This study set out to elucidate the molecular mechanisms by

which RA promotes the TGF-β-induced conversion of naive
CD4+ T cells to the Foxp3+ phenotype. Perhaps surprisingly, we did not observe major effects of RA on the responding cells. There was no enhancement of TGF-β signaling and no complementation of the Treg cell transcriptional signature, and the consequences of RA treatment on gene expression were relatively modest and largely shared between Foxp3-positive and Foxp3-negative cells in the same cultures. Instead, the data point to a rather different mode of action, in which RA counteracts the dampening effects of inhibitory populations whose existence was revealed by cell fractionation and complementation analyses. RA’s activity was entirely mediated by RARα, which provided a useful experimental handle for confirmation of this indirect effect through cocrisscross experiments, in vitro and in vivo.

The use of purified CD4+ cells in the assays allowed us to discern the indirect effect of RA and the impact of counterconversion mediated by CD44hi cells. In contrast, the ability of RA to repress IL-6Rα in naïve T cells, which would reduce their susceptibility to inhibition by IL-6, also suggests the possible existence of direct effects. Although this direct effect appeared of secondary importance in our culture conditions, it may be more prominent in some in vivo situations, where IL-6 made by non-T cells may be more important. RA would thus safeguard TGF-β-induced conversion in several settings.

An indirect effect of RA is compatible with the observation made by most groups that the compound has no effect whatsoever in the absence of TGF-β, which is understandable if it serves to relieve a repression rather than to directly promote conversion. That RA would interact with inhibitory cells is also compatible with the fact that there was substantial variability in the baseline efficacy of conversion (as noted by Benson et al., 2007): most likely, the actual proportion of CD44hi cells in the input population varied between donor pools. The conditions used for these cultures (engagement of CD3 and/or CD28, IL-2 supplementation, DCs) were conducive to cytokine production, and one can conceive of how small experimental variation might affect counterconversion. In fact, the nature of the activating stimulus may modulate the efficacy of RA in enhancing TGF-β-driven Foxp3 expression.

It is currently unclear whether counterconversion may also affect other modes of T cell differentiation elicited by TGF-β. In these cocultures, even with high concentrations of T cell-derived IL-21, we found no evidence for IL-17 production at the protein or mRNA level. Thus, there is clearly more than a dichotomous set of possible outcomes (Foxp3 or IL-17 expression) for naïve T cells stimulated with TGF-β.

The ability of CD4+ Tconv cells to convert to a Foxp3+ phenotype when stimulated in a TGF-β-rich environment has been proposed as a means for achieving a balanced and regulated response, particularly in mucosal areas, such as the GALT, where peaceful coexistence (if not actual tolerance) must be achieved with the intestinal commensal flora. The ability to generate Treg cells when intestinal homeostasis is threatened is thus desirable, but it is also important that the system limit the potential for a “runaway” conversion to Treg cell phenotypes, which would overly suppress needed responses. The existence of a counterconversion pathway would be a logical means for controlling this potential; a balance between TGF-β-fueled drive to conversion and counterconversion activity. In this context, RA would serve as an external modulator of this balance. It is provided by a third-party cell, and one can conceive of the DCs acting as sensors and integrators of local microbial challenge and inflammatory responses, influencing the conversion/counterconversion balance by releasing variable amounts of RA. The particular ability of gut DCs to produce RA (Iwata et al., 2004; Coombes et al., 2007) is clearly in line with such a notion.

Intriguingly, RA has little to no influence on the selection of Treg cells in the thymus, nor on their overall proportions in secondary lymphoid organs, as evidenced by the normal Treg cell populations in Rara-deficient mice. This is compatible with a view in which RA-influenced conversion of conventional CD4+ T cells to a Foxp3hi phenotype represents a focused adaptation, involving only particular reactivities or locations, but overall homeostatic control of Treg cell populations falls under a different control, such as the supply of trophic cytokines.

The dampening of Foxp3 induction by cytokines showed clear synergistic effects. IL-4 was the most active in this respect, but IL-21 and IFN-γ clearly enhanced this ability, and they complemented each other in doing so. This synergy is unusual for IL-4 and IFN-γ, which are usually antagonistic. It will be important to elucidate the signaling pathways involved, but one can imagine complex interactions wherein one cytokine induces the receptor or the signaling cascade downstream of the other.

It should also be pointed out that, although counterconversion can be mediated by cytokines in this synergistic fashion, soluble factors do not account for the whole effect of CD44hi cells. Supernatants from CD44hi cells were never quite as effective as the cells themselves, and inhibition of counterconversion with antibody combinations only incompletely reversed the inhibitory effect. Although these elements could be explained by focused release and action of cytokines or by additional soluble mediators, they also suggest that cell-cell interactions may be involved in counterconversion.

What cells mediate counterconversion? The results indicate that the function resides, at least in part, in CD44hi CD4+ T cells. It does not involve NK cells, a priori attractive candidates because of their ability to produce IL-4 and IFN-γ; sorted NK1.1+ positive cells within the CD44hi fraction had no counterconversion ability (not shown). Similarly, Treg cells themselves did not show activity (a priori an appealing hypothesis in which Treg cells would negatively feed back on their own generation), given that purified Foxp3-GFP+ cells added to the cultures did not influence conversion (not shown). In contrast, counterconversion ability was found in the CD44hi subset of CD8+ T cells, suggesting that the activity resides in populations of antigen-experienced Tconv cells with markers consistent with effector and/or memory cells.

In fairness, although the concept of counterconversion is logical and has operational value, we do not know whether a dedicated “counterconvertor” population exists. Such a label does carry the teleological connotation of a distinct and identifiable population whose role (or one of whose roles) is to maintain the conversion or counterconversion balance. An alternative scenario is one in which there is no such uniquely identifiable population but rather a set of differentiated states that produce a set of cytokines that dampen TGF-β-mediated Foxp3 induction. This effect could be instilled in an antigen- or location-specific manner and perpetuate the dominance of effective T cell responses, by inhibiting the neogeneration of Treg cells, even in locations of...
Figure 5. CD44<sup>hi</sup> Memory CD4 T Cells Restrain TGF-β-Mediated Foxp3 Expression in Naive CD4 T Cells
(A) Naive CD25<sup>−</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup>CD4<sup>+</sup> T cells (0.5 × 10<sup>5</sup>) were activated with anti-CD3 and anti-CD28 beads and TGF-β (10 ng/ml) in the absence or presence of RA (100 nM). These cells were cultured either alone or in combination with CD25<sup>−</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> memory CD4<sup>+</sup> T cells (0.5 × 10<sup>5</sup> memory cells to 0.25 × 10<sup>5</sup> naive cells) for 4 days. For comparison, CD44<sup>hi</sup> memory T cells were also cultured alone (under identical conditions as those for naive cells that were cultured alone). Individual populations were tracked with the use of CD45.1 or CD45.2 congenic markers. Foxp3 expression in congenically marked populations was determined by FACS, and a representative experiment is shown.
(B) FACS analysis for Foxp3 expression in multiple experiments as described in (A) (n = 14–19). Statistically significant differences were determined by Student’s t test.
strong TGF-β exposure. Whether one or several cell types affect counterconversion, it is striking that RA represses the entire inhibitory program.

**EXPERIMENTAL PROCEDURES**

**Mice**
CS7BL/6J (CD45.1 congenic), C57BL/6J, C57BL/6-Il4−/−, and C57BL/6-Il4−/− mice were bred in the SPF Joslin facility or purchased from Jackson Laboratories. Rae-deficient mice (Chapelle et al., 2002a; Chapelle et al., 2002b) were bred in the SPF facility at IGBMC, whereas Foxp3-eGFP reporter mice (Bettelli et al., 2006) and OT-II Rag−/− (CD45.1 congenic) mice were bred in the SPF facility at NIAD.

**Cells**
Cells used for in vitro activation and in vivo transfer were obtained from spleen and lymph nodes or lamina propria of 6- to 8-wk-old mice. Unless otherwise noted, cells were sorted to obtain individual T cell or DC subpopulations.

**Cell Sorting and Flow Cytometry**
For Figures 1 and 2, CD4+CD25− T cells or CD4+Foxp3-eGFP+ T cells and lamina propria DCs (LP DC) or spleen DCs (Sp DC) were isolated as described previously (Sun et al., 2007). For experiments subsetting CD4+ cell types, naive cells were sorted as B220−,CD8−,CD11c−,CD25−,CD4+,CD44−,CD62L+, whereas the “memory” pool was sorted as B220−,CD8−,CD11c−,CD25+,CD4+,CD44+,CD62L+. OT-II Rag−/− CD4+ T cells were sorted as a naive population (as described above but without staining for CD62L). Postactivation analysis assessed CD4, CD45.1, CD45.2, sIg7, CCR9, IL6Rα, Foxp3, IL10, IL-4, or IFN-γ expression by cell-surface and intracellular antibody staining.

**In Vitro Activation**
For conversion assays, T cells were activated with anti-CD3 and anti-CD28 beads (Dynal) at a concentration of one bead per cell in the presence of 20 U/ml recombinant human IL-2 (Peprotech), or 10 ng/ml recombinant TGF-β (Peprotech), for 4 days in a 96-well plate. Naive CD4+ T cells were cultured at a concentration of 0.5 × 10^5 per well, or 0.25 × 10^5 per well when used in coculture assays. Memory CD4+ T cells were seeded at a 2:1 (memory:naive) cell ratio in coculture experiments (unless otherwise noted). Some cultures were also treated with all-trans RA (100 nM unless otherwise noted, cells were cultured with 0.1 × 10^5 purified LP or Sp DCs in the presence of anti-CD3 (1 μg/ml), TGF-β (3 ng/ml), IL-2 (5 ng/ml), and RA (10 or 100 nM) as described previously (Sun et al., 2007).

**In Vivo Conversion Assay**
OT-II Rag−/− CD4+ T cells (0.5–1 × 10^6 per mouse) were transferred i.v. into either Rara−/− or WT littermate controls as described previously (Sun et al., 2007). The drinking water of recipient mice was supplemented with 1.5% ovalbumin and changed every 2 days. Mice were sacrificed 7 days after T cell transfer, and Foxp3 expression in CD45.1+ donor T cells was detected in skin-draining lymph nodes, spleen, mesenteric lymph node, and the lamina propria.

**Microarrays**
RNA was prepared from sorted cell populations as described previously (Trizol; Yamagata et al., 2004). RNA was amplified for two rounds (Messagelamp, Ambion), biotin-labeled (BioArray High Yield RNA Transcription Labeling, Enzo), and purified with the RNasy Mini Kit (QIAGEN). The resulting cRNAs were hybridized to M430 2.0 chips (Affymetrix). All cell populations analyzed were generated in duplicate or triplicate. Raw data were normalized with the RMA algorithm implemented in the “Expression File Creator” module from the GenePattern software package (Reich et al., 2006). Data were visualized with the “Multiplot” module from GenePattern.

**ACCESSION NUMBERS**
The NCBI GEO accession number for the microarray data reported in this paper is GSE13396.

**SUPPLEMENTAL DATA**
Supplemental Data include Supplemental Experimental Procedures and three figures and can be found with this paper online at http://www.immunity.com/S1074-7613(08)00472-X.

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(C) Coculture experiments with congenically marked CD44+/memory or naive T cells were performed in the absence or presence of RA and with different ratios of CD44+ memory T cells to naive T cells (0.5 × 10^6). Cells were activated (as in [A]) and analyzed for Foxp3 expression 4 days later. Data presented are representative of three independent experiments.

(D) Supernatant from CD44+CD25− T cells activated with anti-CD3 and anti-CD28 beads for 48 hr can inhibit Foxp3 expression in naive T cells. Naive T cells were activated with anti-CD3 and anti-CD28 beads and TGF-β (10 ng/ml) in the presence or absence of 100 ul of memory T cell supernatant and analyzed for Foxp3 expression 4 days later.

(E) Naive T cells were activated with anti-CD3 and anti-CD28 beads and the indicated concentrations of RA for 24 hr, then cultured in the presence of TGF-β (10 ng/ml) and the indicated concentration of supernatant from memory T cells for 4 days. Representative FACs data from one of three experiments are shown.

Data are presented as percentage of Foxp3 expression in the test condition relative to that found in naive T cells alone (control).

(F) Memory T cells were activated and cultured in the presence or absence of RA. Supernatants were harvested at different time points and added to naive T cells in the conversion assay. Representative FACs data from one of three experiments is shown. Data are presented as percentage of Foxp3 expression in the test condition relative to that found in naive T cells alone (control).

(G) Crisscross experiments with Rara−/− memory (left panel) or naive (right panel) CD4+ T cells in coculture with congenically marked WT naive (left panel) or memory (right panel) CD4+ T cells were conducted as described in (A). No enhancement of Foxp3 expression was seen when Rara−/− memory CD4+ T cells were cultured with WT naive CD4+ T cells in the presence of RA. A significant enhancement (p < 0.01) of Foxp3 expression was seen when Rara−/− naive CD4+ T cells were cultured with WT memory CD4+ T cells in the presence of RA. Representative FACs plots are shown with the mean and SD from three independent experiments.
Figure 6. Counterconversion Is Mediated by Cytokines and Alleviated by Blocking of IL-4, IFN-γ, and IL-21
(A) Congenically marked CD44hi memory CD4+ T cells were isolated from the coculture assay at 48 hr and processed for microarray analysis. Mean expression (CD44hi memory T cells, x axis) versus FoldChange (CD44hi memory RA versus CD44hi memory T cells, y axis) plot of expression data from memory T cells treated with RA or without RA in the coculture assay. Secreted extracellular factors identified from Gene Ontology (GO) analysis are highlighted in red.
(B) Recombinant cytokines were added individually to cultures of naive CD4+ T cells activated with anti-CD3 and anti-CD28 beads and TGF-β. Cells were harvested at day 4 for testing for Foxp3 expression by FACS. Representative data are shown for one of three or more experiments.
(C) Inhibition of cytokine signaling in coculture experiments using blocking antibodies. Naive and memory coculture experiments were performed in the absence or presence of cytokine-blocking antibodies (5 μg/ml). Cells were harvested at day 4 for testing for Foxp3 expression in congenically marked cells by FACS. Statistically significant differences were determined by Student’s t test.
(D) Recombinant IFN-γ (1 ng/ml), IL-4 (1 ng/ml), and IL-21 (10 ng/ml) were added individually, in pairs, or altogether to cultures of naive CD4+ T cells activated with anti-CD3 and anti-CD28 beads and TGF-β. Cells were harvested at day 4 and tested for Foxp3 expression by FACS. Shown are representative FACS data with average values ± SEM for six individual experiments.

REFERENCES
Figure 7. RARα Signaling Can Indirectly Alter Foxp3 Conversion In Vivo
(A) Foxp3−/− OT-II Rag1−/− CD45.1 CD4+ T cells were sorted and then injected i.v. into Rara−/− mice or WT littermates (0.5–1 × 10^5 cells per mouse). Mice were given water supplemented with ovalbumin (1.5%) for 7 days, after which point they were sacrificed and donor OT-II CD45.1 T cells from various lymphoid organs were analyzed for Foxp3 expression by FACS. Shown are representative FACS plots showing Foxp3 expression in CD45.1+ CD4+ T cells from the mesenteric lymph nodes of either a WT or Rara−/− host.
(B) Summary of Foxp3 expression in donor CD45.1+ OT-II T cells harvested from lamina propria or mesenteric lymph nodes of Rara−/− mice or WT littermate controls from multiple experiments.


