Retinoic acid can enhance conversion of naive into regulatory T cells independently of secreted cytokines

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It has been reported that retinoic acid (RA) enhances regulatory T (T reg) cell conversion by inhibiting the secretion of cytokines that interfere with conversion. This report shows that these conclusions provide a partial explanation at best. First, RA not only interfered with cytokine secretion but also with the ability of these cytokines to inhibit T reg cell conversion of naive T cells. Furthermore, RA enhanced conversion even in the absence of inhibitory cytokines. The latter effect depended on the RA receptor α (RARα) but did not require Smad3, despite the fact that RA enhanced Smad3 expression. The RARα1 isoform was not essential for RA-dependent enhancement of transforming growth factor β–driven conversion, suggesting that conversion can also be mediated by RARα2. Interleukin (IL)-6 strongly reduced RARα expression levels such that a deficiency of the predominant RARα1 isoform leaves too little RARα2 for RA to inhibit the generation of Th17 cells in the presence of IL-6.

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Abbreviations used: RA, retinoic acid; RARα, RA receptor α.

T reg cells play an important role in regulating immunity and have an essential function in preventing fatal autoimmune disease (Sakaguchi et al., 2006; Ziegler, 2006). T reg cells come in at least two flavors, referred to as intrathymically and extrathymically induced T reg cells (Chen et al., 2003; Apostolou and von Boehmer, 2004; Kretschmer et al., 2005; Mucida et al., 2005; Hsieh et al., 2006; Pacholczyk et al., 2006; Sun et al., 2007). Recently, it was shown that retinoic acid (RA), produced by specialized DCs in the gut (Benson et al., 2007; Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007), can enhance the peripheral conversion of naive $CD4^+$ T cells into T reg cells, a process that is dependent on TGF-β–induced signaling both in vitro and in vivo (Chen et al., 2003; Curotto de Lafaille et al., 2004; Fantini et al., 2004; Kretschmer et al., 2005).

Initially, it was hypothesized that RA has a direct effect on the converting cells either by counteracting the negative effect of IL-6 signaling (Mucida et al., 2007) or by preventing the formation of AP-1 (von Boehmer, 2007). In contrast to these models, a more recent analysis of carefully separated T cell subsets concluded that RA elicits its effect through "contaminating" activated $CD44^{hi}$ cells that secrete cytokines in response to antigenic stimulation, and these cytokines in turn prevent the conversion of naive T cells into T reg cells. It was concluded that RA prevented cytokine production by $CD44^{hi}$ cells, thereby enhancing T reg cell conversion (Hill et al., 2008). Independent studies, however, argued for a more direct role of RA on the conversion of T cells (Takaki et al., 2008; Xiao et al., 2008). In a recent exchange of letters (Hill et al., 2009; Mucida et al., 2009), it was proposed that RA directly affects conversion of naive T cells (Mucida et al., 2009) and that this effect could be caused by inhibition of cytokine secretion by naive T cells (Hill et al., 2009). It remains unclear, however, whether the effect of RA on T reg cell conversion is always caused by

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the inhibition of cytokine secretion or whether there are additional mechanisms that are cytokine independent and might involve direct regulation of genes, such as FoxP3, that are expressed in T reg cells.

We have investigated the role of RA in the T reg cell conversion process in more detail by analyzing the contribution of CD44^{hi} cells, titrating co-stimulating CD28 antibodies as well as cytokines, and by addressing molecular pathways involved in the conversion process. The results show that RA can interfere with the negative effect of co-stimulation and certain cytokines on naive T cells rather than directly inhibiting cytokine secretion. We also found that RA enhances T reg cell conversion of naive T cells in the absence of inhibitory cytokines. The latter mechanism depended on the expression of RA receptor α (RARα) but was largely Smad independent, in spite of RA's ability to increase Smad3 but not Smad2 transcription in naive T cells. Of note, deficiency of the RARα1 isoform interferes with the TGF-β– plus IL-6–dependent conversion of Th17 cells but has much less of an effect on T reg cell conversion. This is because of the fact that IL-6 severely reduces RARα expression levels.

RESULTS AND DISCUSSION

Direct impact of RA on T reg cell conversion depends on mode of T cell activation

When CD4$^+$ T cells were depleted of CD44^{hi} cells (Fig. 1 A) and subjected to an in vitro TGF-β–dependent T reg cell conversion assay, the effect of RA on the conversion process was clearly diminished, but not abolished, when cells were cultured with CD3 and CD28 antibodies at a 1:1 ratio (Fig. 1 B). In fact, the magnitude of the RA-enhancing effect depended on co-stimulation because it was reduced when only CD3

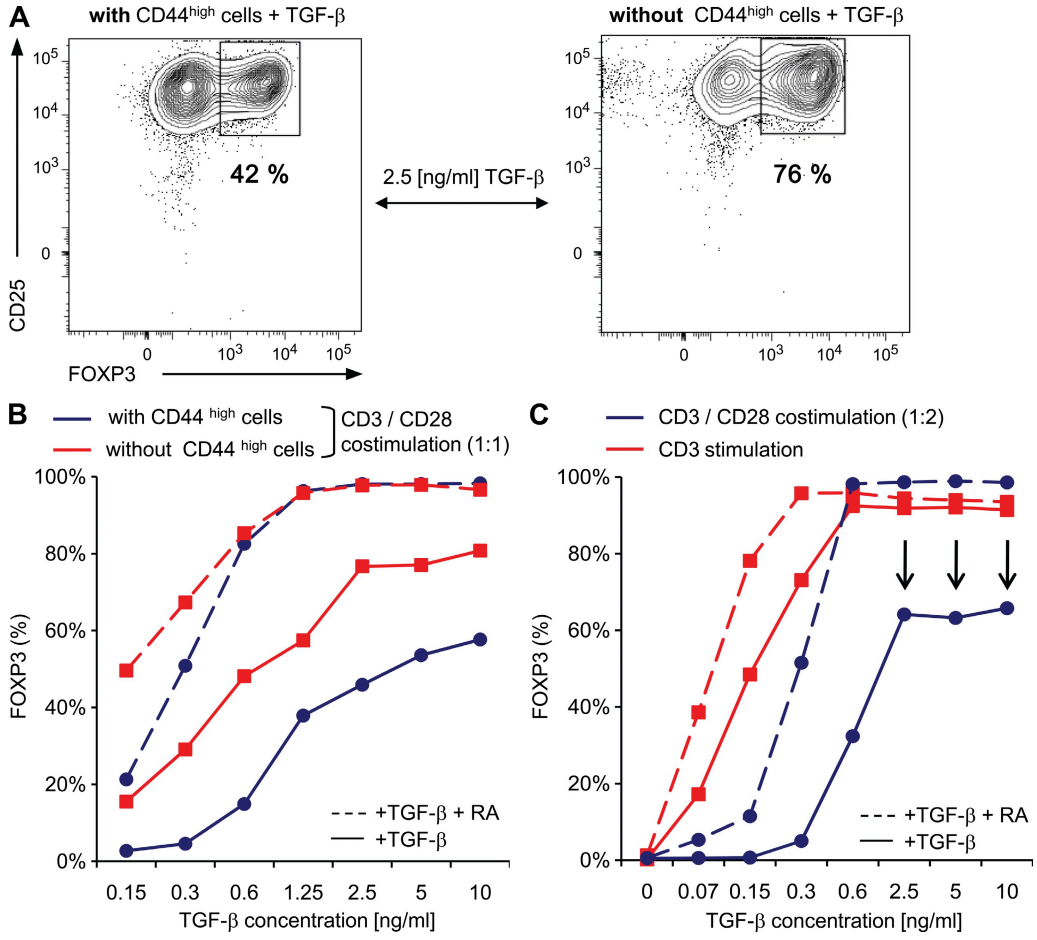

Figure 1. Direct effect of RA on T reg cell conversion by counteracting co-stimulation. (A) CD44^{hi} cells inhibit TGF-β–mediated Foxp3 expression. Comparison of T reg cell conversion of CD4$^+$ naive T cells from Foxp3-GFP reporter mice activated with anti-CD3 and anti-CD28 and 2.5 ng/ml TGF-β in the presence or absence of CD44^{hi} cells. Numbers indicate percentages of Foxp3-expressing cells gated on CD4$^+$ cells. Shown is one experiment representative of six independent experiments. (B) RA enhances Foxp3 expression in purified naive CD4$^+$ T cells over a range of TGF-β concentrations. Continuous lines show the frequency of Foxp3$^+$ cells of cultures in the presence of CD44^{hi} cells (blue) or in the absence of CD44^{hi} cells (red). Dashed lines indicate cultures that contained 2.5 nM RA. Data are from three independent experiments. (C) RA directly counteracts co-stimulation. Naive T cells from Foxp3 reporter mice were cultured with CD3 antibodies alone (red) or CD3 and CD28 antibodies (blue) at a ratio of 1:2 in the presence of the indicated TGF-β concentrations. Dashed lines represent cultures containing 2.5 nM RA. Representative data are shown of three independent experiments.

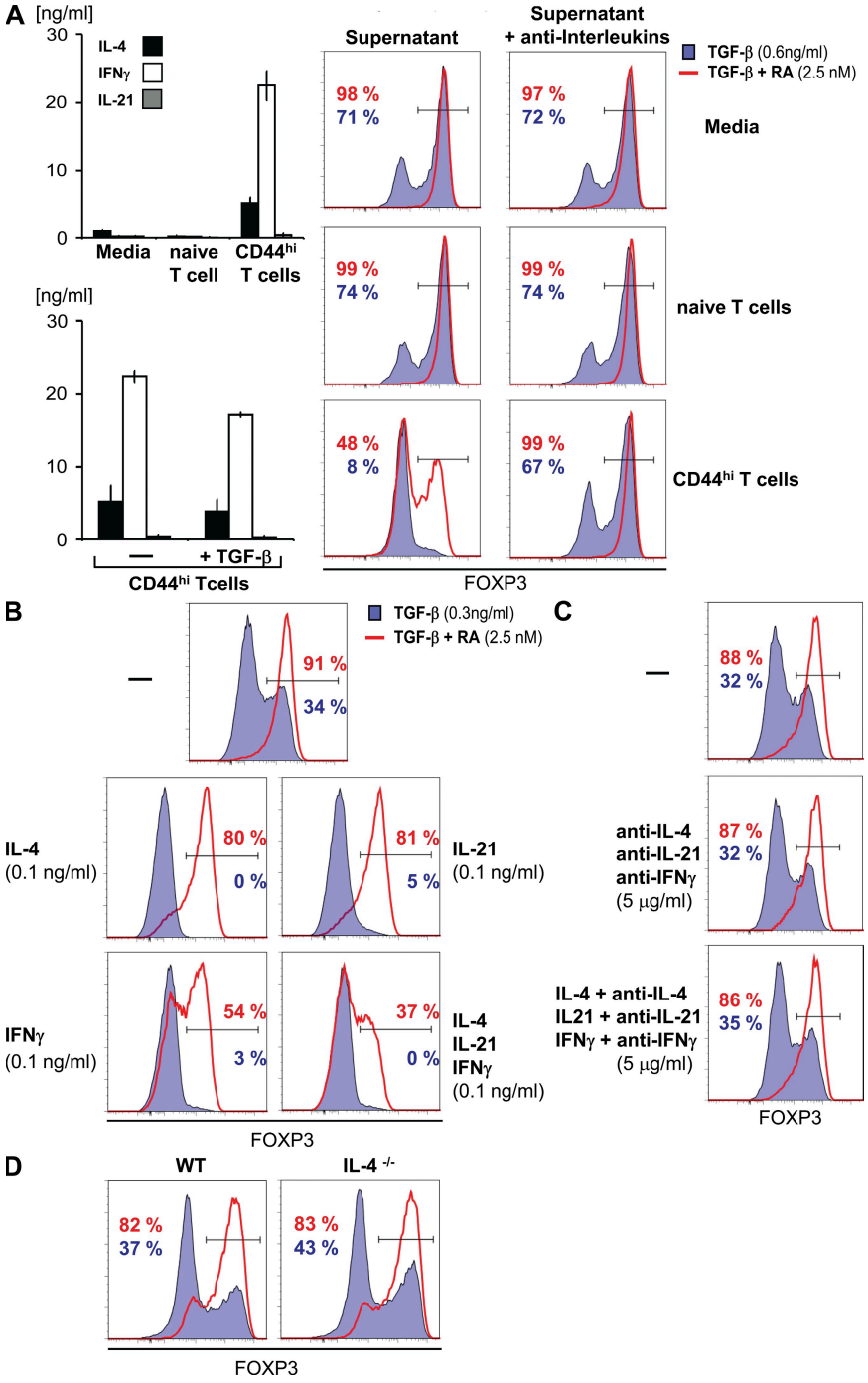

Figure 2. RA enhances T reg cell conversion independently of secreted cytokines and neutralizes the effect of cytokines on naive T cells. (A, left) Cytokines in supernatants of stimulated naive or CD44^{hi} cells. Naive CD4$^+$ T cells or CD44^{hi} cells were activated with anti-CD3 and anti-CD28, and the supernatants were measured after 48 h by ELISA for IL-4, IFN-γ, and IL-21. In some cultures 0.6 ng/ml TGF-β was added. (right) Supernatants of CD44^{hi} but not naive T cells contain cytokines inhibiting T reg cell conversion. Conversion of naive T cells is enhanced by a direct effect of RA: naive CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 and 0.6 ng/ml TGF-β in medium or supernatant from CD44^{hi} or naive CD4$^+$ T cells, and diluted with fresh media at a ratio of 1:1 in the presence (open histogram) or absence (shaded histogram) of 2.5 nM RA. Representative data (means ± SEM) are shown for two independent experiments. (B) RA neutralizes the adverse effect on conversion of various cytokines. 0.1 ng/ml of recombinant IL-4, IFN-γ, and IL-21 were added individually or in combination to cultures of naive CD4$^+$ T cells activated with anti-CD3 and anti-CD28 and 0.3 ng/ml TGF-β in the presence (open histogram) or absence (shaded histogram) of 2.5 nM RA. Shown are representative FACS data from three individual experiments. (C) Antibodies to IL-4, IFN-γ, and IL-21 can neutralize the inhibitory effect of CD44^{hi} cell supernatant on T reg cell conversion. (D) RA enhances conversion of IL-4–deficient naive T cells. Naive CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 and TGF-β in the presence (open histogram) or absence (shaded histogram) of RA. One representative experiment out of three is shown.

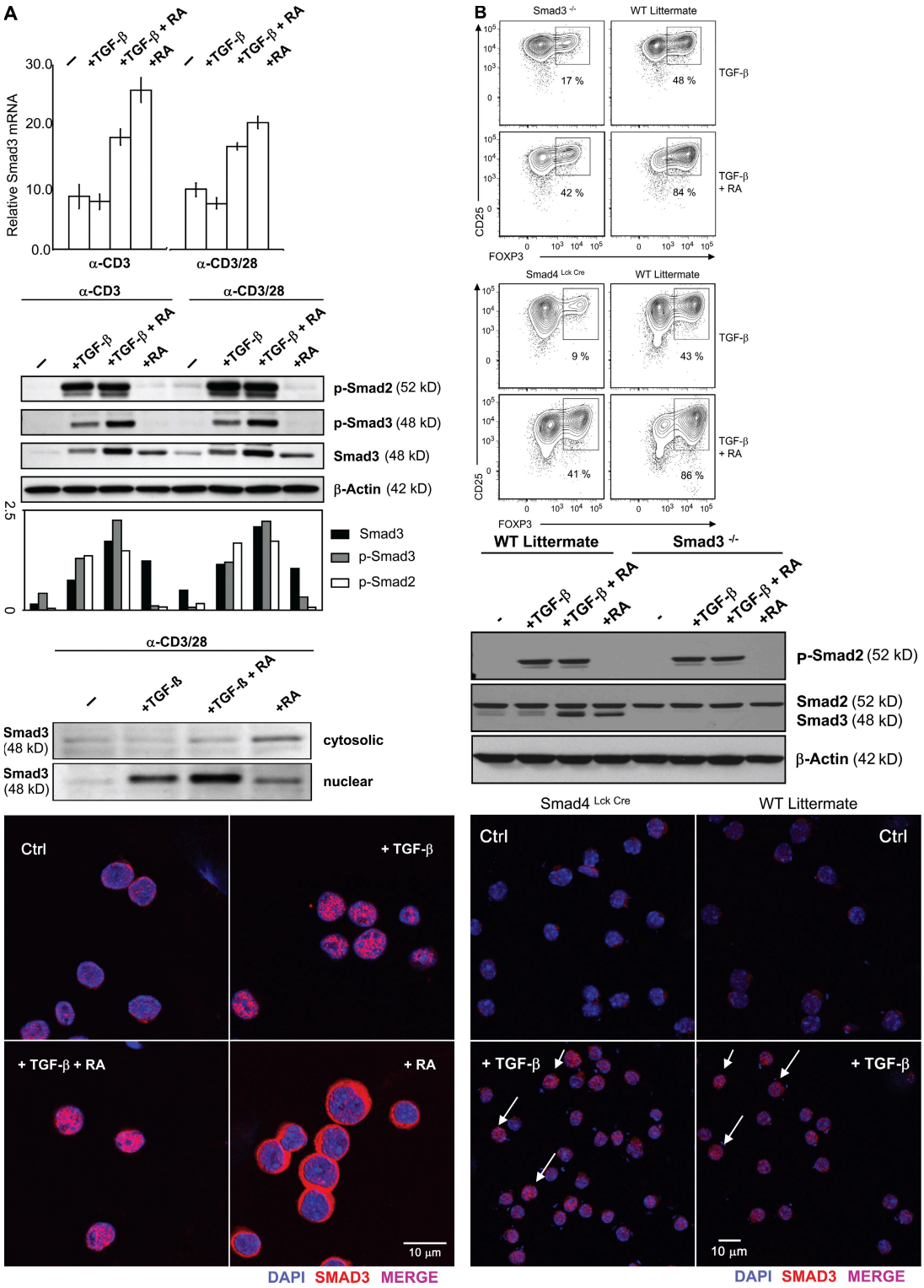

Figure 3. RA-enhanced expression and phosphorylation of Smad3. (A) Naive CD4+ T cells were activated with anti-CD3 alone or anti-CD3 and anti-CD28 for 12 h. (top) Total RNA was isolated, and expression of Smad3 mRNA was determined by quantitative real-time PCR. Data (means ± SEM) are representative of three independent experiments. (middle) Cell lysates were separated by SDS-PAGE gel, and total Smad3, p-Smad3, and p-Smad2 were analyzed by immunoblotting with specific antibodies. For quantification, the ratio of total Smad3/β-actin as well as the ratios of p-Smad3/β-actin and p-Smad2/β-actin were determined by densitometric analysis. Nuclear and cytoplasmatic extracts were analyzed by immunoblotting with specific antibodies. Cells were fixed and stained for confocal analysis by a Smad3 antibody. Data are representative of three to five independent experiments. (bottom) T reg cell conversion of naive CD4+ T cells from Smad3$^{-/-}$ or Smad4$^{-/-}$ or WT control mice activated with anti-CD3/CD28 in the presence of 0.3 ng/ml TGF-β

antibodies were used. Adding CD28 antibodies in excess (2:1) decreased the conversion rate in the absence of RA, and RA could fully reverse this decrease (Fig. 1 C). Thus, depending on the conditions under which naive T cells are stimulated, RA can have a direct effect on the conversion of naive T cells (Mucida et al., 2009), and this becomes more apparent under conditions of strong co-stimulation (Fig. 1; Benson et al., 2007).

RA directly neutralizes the adverse effect of cytokines on T reg cell conversion

These observations could be interpreted to suggest that strong co-stimulation results in cytokine release from the stimulated naive T cells that then interferes with conversion, as suggested previously (Hill et al., 2009). According to this assumption, RA could interfere with the production of cytokines by both $CD44^{lo}$ and $CD44^{hi}$ cells. Alternatively, but not mutually exclusive, one could argue that RA interferes with the effect of cytokines on naive T cells, as postulated by others (Takaki et al., 2008; Xiao et al., 2008). Finally, RA could directly enhance T reg cell conversion independently of cytokine secretion. In fact, it appears that all of these scenarios apply.

To address these issues, $CD44^{hi}$ and naive T cells were stimulated and their supernatants were tested for the capacity to inhibit TGF-β–dependent conversion in the presence or absence of RA. As shown in Fig. 2, inhibitory cytokines were produced by $CD44^{hi}$ but not by naive T cells, and these cytokines could inhibit conversion irrespective of whether the $CD44^{hi}$ cells were stimulated in the presence or absence of TGF-β. IL-4 and IFN-γ could be detected in the supernatants of $CD44^{hi}$ cells, and antibodies to IL-4, IFN-γ, and IL-21 could neutralize the inhibitory effect of the supernatant on T reg cell conversion (Fig. 2 C). Furthermore, RA could reverse the inhibitory effect of IL-4, IFN-γ, and IL-21 on the TGF-β–dependent conversion of naive T cells in a dose-dependent manner (Fig. S2).

These experiments therefore indicate that RA can affect T reg cell conversion in multiple ways. One mechanism involves interfering with the secretion of cytokines by $CD44^{hi}$ cells (Hill et al., 2008), and another involves interfering with the inhibitory effect of cytokines on the TGF-β–dependent conversion of naive T cells. A third mechanism involves enhancement of T reg cell conversion in the absence of any inhibitory effect of secreted cytokines. Indeed, we showed that, contrary to prior assumptions (Hill et al., 2009), naive T cells do not produce any inhibitory cytokines when cultured with anti-CD3/28 in the presence of TGF-β. Conceivably, in the latter two scenarios RA could operate by the same mechanism, for instance by directly enhancing Foxp3 expression.

In conclusion, there is a direct effect of RA on Foxp3 expression that does not involve reversing the inhibitory effect of secreted cytokines. IL-4 in particular has been shown to lead to a marked decrease of Foxp3 expression, and this cytokine could conceivably be secreted at low levels by naive T cells (Hill et al., 2008; Takaki et al., 2008). However, we show that RA also enhances the conversion of IL-4–deficient naive T cells into $FoxP3^+$ T reg cells (Fig. 2 D).

RA increases Smad3 but nevertheless enhances T reg cell conversion independently of Smad3

The data in Figs. 1 and 2 indicate that RA can directly affect the conversion of naive T cells into $Foxp3^+$ T reg cells in the absence of secreted cytokines. However, these experiments did not address the molecular mechanism by which this was achieved. Because RA cooperates with TGF-β in T reg cell conversion, we analyzed the impact of RA on TGF-β–dependent signaling pathways, especially Smad proteins, because the Smad3 gene contains a putative single RA response element (Schug et al., 2007).

Indeed, RA increased Smad3 RNA and protein levels in naive T cells and, in concert with TGF-β, yielded more phosphorylated Smad3 in the nucleus (Fig. 3 A). Although these data could provide a mechanism to explain how RA cooperates with TGF-β in T reg cell conversion, experiments with Smad-deficient mice indicated that this pathway does not play an essential role in RA-dependent T reg cell conversion (Fig. 3 B). In contrast, TGF-β–dependent T reg cell conversion is much more Smad dependent: in the presence of TGF-β, Smad3-deficient cells converted less efficiently than did WT cells, with the percentage of $Foxp3^+$ cells dropping from 48 to 17%. In spite of this decrease, RA could still strongly enhance the conversion of Smad3-deficient naive T cells into Foxp3-expressing T reg cells.

Smad4 is essential for shuttling phosphorylated Smad3 or Smad2 into the nucleus, and for stabilizing Smad2/3 DNA complexes (Massagué et al., 2005). Consistent with this role of Smad4, TGF-β–dependent conversion was strongly reduced in Smad4-deficient versus WT naive T cells (9 vs. 43%). Again, RA considerably enhanced the conversion of Smad4-deficient naive T cells into $FoxP3^+$ T reg cells (Fig. 3 B). These results indicate that TGF-β–dependent in vitro conversion depends largely on Smad proteins, whereas RA-dependent enhancement of this conversion does not. Of note, even in Smad4-deficient mice, some Smad3 does enter the nucleus in the presence of TGF-β, which could explain why the Smad4 deficiency does not entirely abolish T reg cell conversion in vitro and has little effect on the proportion of T reg cells in various lymphoid organs in vivo

or TGF-β plus 2.5 nM RA. (B, top) Representative FACS plots are shown (n = 3 independent experiments). (middle) Cell lysates of naive $CD4^+$ cells from $Smad3^{-/-}$ and WT control mice were cultured for 12 h (as in A, bottom) and separated by SDS-PAGE gel, and total Smad3 and Smad2 were detected by immunoblotting. Representative data out of four independent experiments are shown. (bottom) Naive $CD4^+$ T cells from $Smad4^{-/-}$ or WT control mice were fixed and stained by Smad3 antibodies and analyzed by confocal microscopy. The arrows indicate translocation of Smad3 into the nucleus. Representative data are shown (n = 3 independent experiments).

(Yang et al., 2008), with the possible exception of the gut (Fig. S3). However, in vivo conversion could be largely Smad independent.

Role of RARα1 and RARα2 isoforms

The final analysis addressed the role of RARα in RA-enhanced conversion of naive T cells in the absence of CD44^{hi}-activated T cells. As shown in Fig. 4, RA-enhanced conversion was abolished in RARα-deficient naive T cells, whereas there was much less of an effect in the absence of RARα1 alone. This indicates that both RAR isoforms can mediate the RA-dependent enhancement of T reg cell conversion. In contrast, the negative effect of RA on TGF-β– and IL-6–dependent conversion of naive T cells into Th17 cells was much more dependent on the RARα1 isoform. Both RARα and RARα1 deficiency completely blocked the negative impact of RA on Th17 conversion (Fig. 4 A). When analyzing total RARα expression in cells cultured in the absence or presence of IL-6, it became clear that IL-6 strongly reduced RARα expression (Fig. 4 B). Thus, a deficiency of the predominant RARα1 isoform leaves very little RARα2 expressed in cells cultured in the presence of IL-6 (Fig. 4 B). It is therefore possible that RARα1 and perhaps also RARα2 can mediate RA-dependent enhancement of T reg cell conversion and the inhibitory effect of RA on the generation of Th17 cells. However, there is too little RARα2 left in RARα1-deficient cells in IL-6 containing cultures to reach definitive conclusions about RARα2.

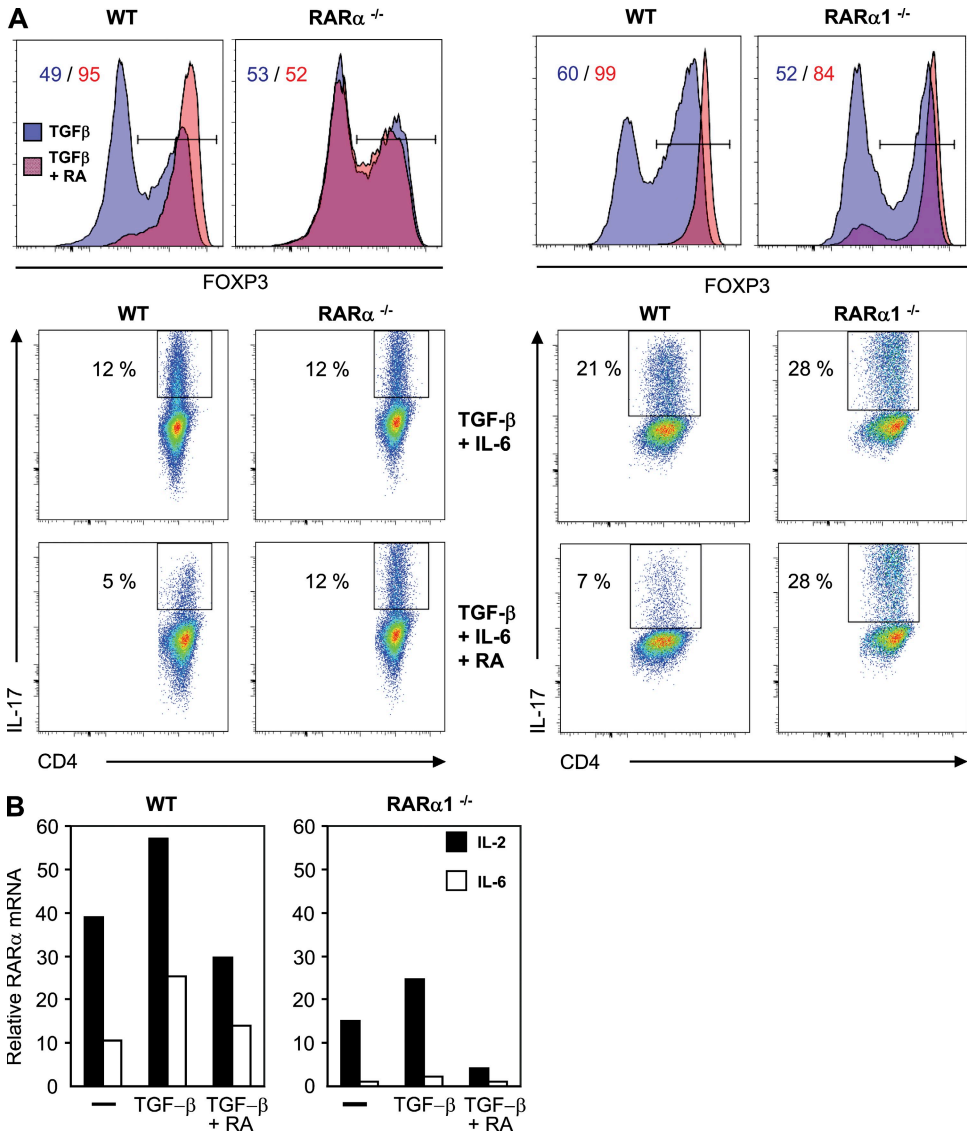

Figure 4. RARα deficiency but not RARα1 deficiency blocks T reg cell conversion. (A) Naive CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 for 3 d under the indicated conditions, and T reg cell or Th17 conversion was analyzed by intracellular staining of Foxp3 or IL-17 (percentages are indicated). Representative FACS plots are shown of three independent experiments. (B) IL-6 reduces the RARα content of naive T cells. Naive CD4$^+$ T cells were activated as in A. After 24 h, cells were collected for the determination of RARα by TaqMan quantitative PCR. Data are representative of three independent experiments.

The data presented in this report confirm that "contaminating" CD44^{hi}-activated T cells can have an influence on the RA-dependent enhancement of TGF-β–dependent T reg cell conversion. This is particularly true under conditions of limited co-stimulation, where most of the RA-dependent enhancement may be caused by interference with cytokine production by CD44^{hi} cells (Hill et al., 2008). However, even under these conditions, RA can also directly interfere with the inhibitory impact of exogenous cytokines on T reg cell conversion of naive T cells. Furthermore, RA can interfere directly with the negative impact of co-stimulation on T reg cell conversion of naive T cells and can enhance T reg cell conversion of naive T cells independently of secreted cytokines.

When analyzing the role of Smad proteins in TGF-β– and RA-dependent conversion, we find that RA can increase Smad3 expression and the accumulation of phosphorylated Smad3 in the nucleus, in accord with previous findings (Xiao et al., 2008). However, this pathway was not essential for RA-enhanced, TGF-β–dependent conversion because the enhancing effect of RA was still discernible in Smad3-deficient naive T cells. On the other hand, in vitro conversion of naive T cells in response to TGF-β alone is dependent on Smad proteins. It is of interest that Smad4-deficient mice do not exhibit abnormalities in the proportion of T reg cells in various lymphoid organs, with the possible exception of the gut (Fig. S3). This could indicate that other mechanisms, such as Smad4-independent nuclear localization of Smad3, could compensate for the Smad4 deficiency in vivo or that the generation of the intestinal T reg cells is more Smad dependent than is the accumulation of T reg cells in other lymphoid organs. Irrespective of in vivo conversion mechanisms, TGF-β–dependent in vitro conversion is largely dependent on Smad proteins, whereas RA-dependent enhancement of conversion is not. The direct enhancement of T reg cell conversion of naive T cells by RA in the absence of inhibiting cytokines is entirely dependent on RARα and can be mediated by both receptor isoforms. IL-6 reduces the total RARα content of naive T cells such that in the absence of the predominantly expressed RARα1 isoform there is too little RARα2 left to address its role in inhibiting the generation of Th17 cells.

Presently, we have only limited insight into how RA could enhance T reg cell conversion and interfere with Th17 conversion in an RARα-dependent manner. It has been suggested that RARα can regulate Foxp3 expression by binding to regulatory sequences in the Foxp3 gene, thereby counteracting negative effects of cytokine-induced STAT proteins (Takaki et al., 2008) on Foxp3 expression. However, it is also possible that the effect of RARα is more indirect, for instance through interfering with the function of AP-1 (Nicholson et al., 1990; Salbert et al., 1993), which could negatively affect Foxp3–NFAT complexes required for Foxp3-dependent gene regulation (Wu et al., 2006; von Boehmer, 2007).

MATERIALS AND METHODS

Mice. C57BL/6J, C57BL/6-Il4$^{-/-}$ mice were purchased from the Jackson Laboratory. RARα-deficient mice were provided by J. Hill (Harvard Medical School, Boston, MA). The mouse was originally generated in the Chambon Laboratory (Chapellier et al., 2002). RARα1-deficient mice were provided by P. Li (Li et al., 1993), and Foxp3-GFP reporter mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY; Fontenot et al., 2005). Smad3-deficient mice were provided by C. Stuelten. Founder animals for the Smad3-BALB/c colony were a gift from L.W. Bach (Aarhus University Hospital, Aarhus, Denmark) and were bred at the facility at the National Cancer Institute, whereas Smad4$^{Lck\ Cre}$ mice were provided by B.-G. Kim. All mice were maintained in a pathogen-free facility, and all procedures were in accordance with the approved protocols and guidelines of the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

Cell sorting and flow cytometry. mAbs specific for CD4 (RM4-5), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), Foxp3 (FJK-16s), and IL-17 were purchased from BD or eBioscience and were used as biotin, FITC, PE, PerCP-Cy5.5, allophycocyanin (APC), or Pacific blue conjugates. For Fig. 1 (A and B), CD4$^+$CD44^{lo}CD62L^{hi}CD25$^-$Foxp3-GFP$^-$ T cells (without CD44^{hi} cells) or CD4$^+$CD62L^{hi}CD25$^-$Foxp3-GFP$^-$ T cells (with CD44^{hi} cells), T cells (Sun et al., 2007) were sorted from spleens and lymph nodes of 6–8-wk-old mice using a cell sorter (FACSAria; BD). For all other experiments, highly purified naive CD4$^+$ T cells were FACS sorted as CD4$^+$CD44^{lo}CD62L^{hi}CD25$^-$Foxp3-GFP$^-$ T cells (Foxp3-GFP reporter mice) or CD4$^+$CD44^{lo}CD62L^{hi}CD25$^-$ T cells (Fig. S1).

In vitro activation and conversion assays. For T reg cell conversion assays, T cells were activated with plate-bound anti-CD3 alone or together with anti-CD28 antibodies at a concentration of 5 μg/ml in the presence of 100 U/ml of recombinant mouse IL-2 (PeproTech) for 3 d in a 96-well flat-bottom plate. Naive T cells were cultured at a concentration of 0.5×10^5 cells/well. Conversion assays for Western blot analysis were performed in 24-well flat-bottom plates with 10^6 naive T cells per well. Some cultures were also treated with 2.5 nM of all-trans RA (Sigma-Aldrich); the doses of recombinant TGF-β and recombinant mouse IL-4, IL-21, or IFN-γ indicated in the figures (at the indicated concentrations; PeproTech and R&D Systems); or blocking antibodies specific for 5 μg/ml IL-4, IL-21, or IFN-γ (R&D Systems). For Fig. 2 A, supernatants were harvested after 48 h from cultures activated with anti-CD3 and anti-CD28, and the concentration of IL-4, IL-21, and IFN-γ was analyzed by a mouse ELISA set (OptEIA; BD). For further experiments, the supernatant was diluted with fresh media at a ratio of 1:1. For Th17 conversion assays, 5×10^5 sorted naive T cells were stimulated with plate-bound anti-CD3/CD28 antibodies in the presence of 30 ng/ml IL-6 and IFN-γ blocking antibody. In some cultures, 1 ng/ml of recombinant TGF-β and 2.5 nM RA were added. In all cases, cultures were examined by FACS for expression of GFP (Foxp3-GFP reporter), or intracellular staining for Foxp3 or IL-17 was performed.

RNA isolation, real-time PCR, and immunoblotting. Total RNA was prepared from cells cultured for 12 h in the T reg cell conversion assay using the RNeasy kit (QIAGEN) followed by DNase digestion (QIAGEN). cDNA was synthesized from total RNA using Superscript II RT and oligo(dT) (Invitrogen) according to the manufacturer's recommendations. Real-time RT-PCR was performed on a thermal cycler (ABI PRISM; Applied Biosystems) using SYBR green PCR core reagents (Applied Biosystems). Oligonucleotide primer sequences for Smad3 and HPRT1 were as follows: Smad3-F, 5′-CTGGGCCTACTGTCCAATGT-3′; Smad3-R, 5′-CGATGTAGTAGAGCCGCACA-3′; Hprt1-F, 5′-AGTGTTGGATACAGGCCAGAC-3′; and Hprt1-R, 5′-CGTGATTCAAATCCCTGAAGT-3′. TaqMan quantitative PCRs were performed with TaqMan gene expression assays (Applied Biosystems) for GAPDH (Mm99999915_g1) and RARα (Mm00436264_m1). For Western blot analysis, cells were lysed after 12 h in RIPA buffer or for nuclear and cytoplasmatic extracts by Ne-per (Thermo Fisher Scientific) according to the manufacturer's instructions, supplemented with protease inhibitor cocktail (Sigma-Aldrich). 20-μg lysates were separated on 4–20% SDS-PAGE and transferred onto nitrocellulose membrane (Millipore). Nonspecific binding was blocked by incubation in blocking

buffer (5% nonfat milk in TBST), followed by incubation with the primary antibodies and the appropriate horseradish peroxidase–conjugated secondary antibodies diluted in blocking buffer. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (GE Healthcare). The specific antibodies against Smad2/3, Smad3, p-Smad3, and p-Smad2, as well against β-actin, were purchased from Cell Signaling Technology.

Immunofluorescence and confocal microscopy. Cytospins of T cells were prepared, and fixation of cells was performed using 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized using 0.1% Triton X-100 solution on ice. Blocking was done using 3% BSA at room temperature after incubation with primary rabbit anti–mouse Smad 3 antibody (Cell Signaling Technology) overnight. Secondary antibody anti–rabbit Alexa Fluor 546 was performed for 1 h at room temperature, and cells were counterstained for DAPI. Mounting of samples was performed using the ProLong Antifade Kit (Invitrogen) according to the manufacturer's instructions. Images were taken by an upright confocal microscope (LSM 510 META; Carl Zeiss, Inc.).

Online supplemental material. Fig. S1 shows an overview of the FACS sort strategy and input population for in vitro studies. Fig. S2 shows that RA could reverse the adverse effect of IL-4, IFN-γ, and IL-21 on the TGF-β–dependent conversion of naive T cells in a dose-dependent manner, and Fig. S3 shows the proportion of $CD4^+CD25^+$ $Foxp3^+$ T cells in the lamina propria, thymus, spleen, or mesenterial lymph nodes from WT littermate controls and $Smad3^{-/-}$ or $Smad4^{Lck\ Cre}$ mice by intracellular FACS staining of Foxp3. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090639/DC1.

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