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Vitamin A Metabolites Induce Gut-Homing FoxP3$^+$ Regulatory T Cells[1]

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In this study, we report a novel biological function of vitamin A metabolites in conversion of naive FoxP3$^-$ CD4$^+$ T cells into a unique FoxP3$^+$ regulatory T cell subset (termed "retinoid-induced FoxP3$^+$ T cells") in both human and mouse T cells. We found that the major vitamin A metabolite all-*trans*-retinoic acid induces histone acetylation at the *FoxP3* gene promoter and expression of the FoxP3 protein in CD4$^+$ T cells. The induction of retinoid-induced FoxP3$^+$ T cells is mediated by the nuclear retinoic acid receptor α and involves T cell activation driven by mucosal dendritic cells and costimulation through CD28. Retinoic acid can promote TGF-β1-dependent generation of FoxP3$^+$ regulatory T cells but decrease the TGF-β1- and IL-6-dependent generation of inflammatory Th17 cells in mouse T cells. Retinoid-induced FoxP3$^+$ T cells can efficiently suppress target cells and, thus, have a regulatory function typical for FoxP3$^+$ T cells. A unique cellular feature of these regulatory T cells is their high expression of gut-homing receptors that are important for migration to the mucosal tissues particularly the small intestine. Taken together, these results identify retinoids as positive regulatory factors for generation of gut-homing FoxP3$^+$ T cells. ***The Journal of Immunology,*** **2007, 179: 3724–3733.**

Forkhead box P3 transcription factor (human FOXP3/mouse FoxP3; hereafter called FoxP3) is the cell lineage-determining master transcription factor for a regulatory T cell linage commonly known as "CD4$^+$CD25$^+$ regulatory T cells"; FoxP3$^+$ T cells can suppress the proliferation and/or functions of various immune cells (1). The target cell types that are known to be suppressed by FoxP3$^+$ T cells include CD4$^+$ T cells, CD8$^+$ T cells, NKT cells, dendritic cells (DCs),[4] monocytes/macrophages, B cells, and NK cells (2). CD4$^+$ FoxP3$^+$ T cells are generated in the thymus (natural FoxP3$^+$ cells) (3–5), but they can be induced from naive T cells in response to foreign Ags in secondary lymphoid tissues (induced FoxP3$^+$ cells) in a manner similar to the generation of conventional memory and effector T cells (6, 7). Naive FoxP3$^+$ T cells, made in the thymus, circulate and mainly migrate to secondary lymphoid tissues (8, 9). Upon Ag priming, FoxP3$^+$ T cells acquire tissue-specific homing receptors in an accelerated manner to efficiently migrate to various nonlymphoid tissue sites (8, 10, 11). FoxP3$^+$ regulatory T cells play critical roles in prevention of autoimmune diseases and hyperimmune responses as evidenced by X-linked autoimmunity-allergic dysregulation syndrome caused by nonfunctional mutations in the *FoxP3* gene (12–14). Tumor cells and some pathogens can hijack the suppressive functions of FoxP3$^+$ regulatory T cells to evade the host immune responses (15–17). Although it has been reported that gut-homing regulatory T cells are present in the intestinal mucosa in humans and mice (8, 18), the factors that regulate the generation of these gut-homing FoxP3$^+$ regulatory T cells have not been identified.

It is well-established that vitamin A and its metabolites (retinoids) play important roles in regulation of immunity. Retinoids enhance the numbers and effector functions of various immune cell types (19–24). Vitamin A is required for Ab responses to T-dependent and bacterial polysaccharide Ags (25, 26), prevention of activation-induced T cell apoptosis (27, 28), and normal phagocytic functions (29, 30). It is also reported that retinoids are required for B cell Ig switch to IgA (24, 31). The DCs of gut-associated lymphoid tissues can produce retinoic acid from retinol (30) and up-regulate the gut-homing receptors CCR9 and $\alpha_4\beta_7$ on B cells and T cells (32–35). In contrast, vitamin A and retinoids suppress inflammatory responses and tissue damage (36). Supplementation with vitamin A suppressed inflammation in experimental autoimmune encephalomyelitis (20, 37, 38). Moreover, vitamin A supplementation in animals decreased serum concentrations of inflammatory cytokines such as TNF-α and IL-1 but increased suppressive cytokines such as IL-10 (39, 40). Thus, there is a strong body of evidence that vitamin A and retinoids have immune regulatory functions.

In an effort to find a cellular link between vitamin A and regulation of immune responses and to gain insights into generation of gut-homing FoxP3$^+$ regulatory T cells, we investigated the function of retinoic acid in induction of FoxP3$^+$ regulatory T cells. We found a novel function of retinoic acid in differentiation of naive FoxP3$^-$ T cells into a gut-homing FoxP3$^+$ regulatory T cell subset.

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[4] Abbreviations used in this paper: DC, dendritic cell; ATRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; PP, Peyer's patch; ChIP, chromatin immunoprecipitation; SEB, staphylococcal enterotoxin B; 7-AAD, 7-aminoactinomycin D; h, human; m, murine; RXR, retinoid X receptor; DEAB, diethylaminobenzaldehyde; PLN, peripheral lymph node.

Materials and Methods

Cell preparation and culture

Cord blood mononuclear cells were prepared by density gradient centrifuge on Histopaque 1077 (Sigma-Aldrich). T cells were enriched from cord blood by a sheep-RBC rosetting method (41). $CD4^+CD25^-$ T cells (>98% pure; $FoxP3^+$ cell contamination <0.1%) were isolated by the $CD4^+$ T cell isolation kit followed by depletion of $CD25^+$ T cells (Miltenyi Biotec). T cells were cultured with T cell activators (PHA, 5 μg/ml; or immobilized anti-CD3 and soluble anti-CD28) and IL-2 (25 or 100 U/ml) in the presence or absence of all-*trans*-retinoic acid (ATRA; Sigma-Aldrich), Ro41-5253 (retinoic acid receptor α (RARα)-specific antagonist; Biomol), AM-580 (RARα-specific agonist; Biomol), and/or methoprenic acid (a pan-retinoid X receptor (RXR) agonist; Biomol) for 3 days and further cultured in the presence of IL-2 (25 or 100 U/ml) for an additional 3–9 days for differentiation and resting. Generally, 25 U/ml IL-2 was used to demonstrate the FoxP3 induction activity of ATRA (Figs. 1, 2, 3 and 4), and 100 U/ml IL-2 was used for the functional tests (suppression, killing, and migration) of the retinoid-induced $FoxP3^+$ T cells (Figs. 2, 5–7).

Mouse $CD4^+$ T cells were isolated from splenocytes of BALB/c or AKR/J (from The Jackson Laboratory) with the $CD4^+$ T cell isolation kit (Miltenyi Biotec). The $CD4^+$ T cells were incubated with PE-conjugated Abs to CD8, CD19, CD25, CD44, and CD69 (from BioLegend or eBioscience) and then with anti-PE beads (Miltenyi Biotec) to isolate naive ($CD4^+CD25^-CD44^-CD69^-$) T cells by an autoMACS separator (Miltenyi Biotec).

Mouse $CD11c^+$ DCs were isolated from spleen or intestine (Peyer's patch (PP) and small intestinal lamina propria) of AKR/J mice. The tissues were digested with 2 mg/ml collagenase (type 3; Worthington) for 45 min at 37°C. $CD11c^+$ DCs were positively selected with anti-CD11c-PE Ab (BD Biosciences) and anti-PE beads (Miltenyi Biotec) using an AutoMACS. The purity of $CD11c^+$ cells was ~90% and free of $FoxP3^+$ T cells. The use of human blood and animals for this study has been approved by the institutional review boards at Purdue University.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed according to the manufacturer's protocol with modifications (Millipore). A total of 5–8 $\times$ 10^6 T cells were cross-linked for 10 min with 1% formaldehyde and lysed to prepare them for sonication. Sonication was optimized to generate chromosome fragments of 300–800 bp and immunoprecipitated overnight with polyclonal rabbit Ab specific for acetylated histone H4 (Millipore). A control rabbit Ab was used as a negative control for the anti-H4 Ab. The DNA-histone complexes were incubated for 7 h with 0.2 M sodium chloride at 65°C to release histones from DNA. Purified and input DNA fragments were amplified 33 cycles with PCR and separated on agarose gels. The primers used for the ChIP assay were 5′-CCC ATC CAC ACA TAG AGC TTC-3′ and 5′-CAT TGA TAC CTC TCA CCT CTG TG-3′ for the proximal part (−280 to −501 bp from the transcription start site) of the human FoxP3 promoter and 5′-GCT TGA CTA CTT ATA CCT CCG T-3′ and 5′-CCT AAA TCC TTG GAA ACT GG-3′ for the distal part (−1009 to −1215 bp from the transcription start site) of the human FoxP3 promoter. The *β-globin* gene was amplified for internal controls using the two primers: 5′-ACA GTT AGT GGT AGT GAT TCA CAC AGC A-3′ and 5′-GGG CTT CTG TTG CAG TAG GGA AGA AAG TG-3′.

Expression of trafficking receptors and other Ags by $FoxP3^+$ and $FoxP3^-$ T cells

Human T cells were stained with Abs to CCR4 (clone 205410; R&D Systems), CCR5 (clone 45531.111; R&D Systems), CCR6 (clone 53103.111; R&D Systems), CCR7 (clone 150503; R&D Systems), CCR9 (Millennium Pharmaceuticals), CXCR3 (clone 49801.111; R&D Systems), CXCR4 (clone 44717.111; R&D Systems), CXCR5 (clone 51505.111; R&D Systems), CXCR6 (clone 56811.111; R&D Systems), integrin β_7 (FIB504; BD Biosciences), $\alpha_4\beta_7$ (Act-1; Millennium Pharmaceuticals), cutaneous lymphocyte Ag (HECA-452; BD Biosciences), or mouse control IgG2b (Caltag Laboratories). Cells were further stained with biotinylated horse anti-mouse IgG (H+L) Ab (Vector Laboratories) for 20 min, followed by staining with PerCP-streptavidin (BD Biosciences) and Abs to CD25 and CD4. The cells were further stained with an Ab to human FoxP3 (clone 236A/E7; eBioscience) according to the manufacturer's protocol. Mouse T cells were stained with Abs to CCR7 (clone 4B12; BioLegend), CCR9 (clone 242503; R&D Systems) and $\alpha_4\beta_7$ (LPAM-1; eBioscience) or with an Fc fusion protein of CCL27 as previously described (8). The mouse T cells were further stained with biotinylated 2° Abs and allophycocyanin-streptavidin (BD Biosciences). Finally, the cells were stained with an Ab to mouse FoxP3 (FJK-16s; eBioscience) according to the manufacturer's protocol. Stained cells were acquired on a FACSCalibur. Intracellular staining for cytokine production was performed as described previously (42).

Immunohistochemistry

Cultured T cells were spun down on glass slides, dried, and fixed in cold acetone. Slides were blocked with 10% horse serum for 20 min at room temperature. Slides were stained with the Ab to FoxP3 (236A/E7) and, then, with biotinylated horse anti-mouse IgG (H+L) Ab, followed by staining with FITC-streptavidin. The slides were further stained with PE-labeled Abs to CD4 (S3.5) or granzyme A (CB9) and Hoechst 33342. Slides were examined with a Nikon E400 microscope equipped with epifluorescence.

Assessment of target cell suppression and killing by retinoid-treated T cells

Cord blood $CD4^+CD25^-$ T cells (responders, 10^5 cells/well) and indicated cultured T cells (suppressors) were cocultured in 96-well plates for 5 days at indicated ratios in the presence of PHA (5 μg/ml) and irradiated $CD4^-$ cord blood cells as APCs (10^5 cells/well). Cells were further incubated with 1 μCi/well [^{3}H]thymidine for 8 h, and [^{3}H]thymidine incorporation was measured by a β scintillation counter (Packard Top Count Microplate Scintillation Counter; Packard Instruments). Cell-killing assay was performed as described by others with modification (43). For the cell killing assay, control and ATRA-treated suppressor cells were prepared from cord blood $CD4^+CD25^-$ T cells as described above, and target B cells were isolated by SRBC rosetting followed by CD4 T cell depletion ($CD19^+$ cell purity >99%) as previously described (41). To sensitize for cell killing, B cells were activated for 2 days with PHA and stained with CFSE followed by centrifugation on Histopaque 1077 to remove dead cells. Suppressor cells and target B cells were cocultured at a 10:1 ratio in round-bottom 96-well plates. The superantigen staphylococcal enterotoxin B (SEB; 1 μg/ml; Sigma-Aldrich) was added to stimulate the T cells. EGTA was used at 4 mM to block the perforin-dependent cell-killing pathway. Four hours later, cells were harvested and stained with 7-aminoactinomycin D (7-AAD; final 0.5 μg/ml) immediately before flow cytometric detection of dead ($CFSE^+$7-AAD^+ or $CFSE^+FSC^{low}$) target cells.

In vitro chemotaxis

In vitro chemotaxis was performed as described previously (10). Human and mouse CCL25 proteins were purchased from R&D Systems. A total of 5 $\times$ 10^5 T cells in 100 μl were placed in each transwell insert (5-μm pore, 24-well format; Corning Costar). Transwell inserts were placed in 24-well plates containing 600 μl of chemotaxis medium (RPMI 1640 with 0.5% BSA) with indicated chemokines. Cells were allowed to migrate for 3 h in a 5% CO_2 incubator at 37°C. After chemotaxis, cells that migrated to the lower chambers were harvested and stained with Abs to CD4 (clone S3.5; Caltag Laboratories) and human FoxP3 according to the manufacturer's protocol. Stained cells were acquired on a FACSCalibur, and the data were analyzed with the CellQuest program (BD Biosciences).

Induction of retinoid-induced mouse $FoxP3^+$ T cells in vitro or in vivo

The naive human $CD4^+$ ($CD25^-CD44^-CD69^-$) T cells were cultured for 5 days with Con A (2.5 μg/ml, Sigma-Aldrich) in the presence of human (h) IL-2 (50 U/ml) in complete RPMI 1640 medium (10% FBS; HyClone) containing hTGF-β1 (0.5 or 5 ng/ml; R&D Systems) and/or ATRA (10 nM). The cells were harvested and stained for the FoxP3 expression with an anti-FoxP3 Ab (FJK-16s; eBioscience). For induction of retinoid-induced mouse $FoxP3^+$ T cells, $CD11c^+$ DCs (1 $\times$ 10^5) were cocultured for 5 days with purified syngeneic naive ($CD4^+CD25^-CD44^-CD69^-$) T cells (1 $\times$ 10^5) in the presence of the superantigen SEB (5 μg/ml) with or without diethylaminobenzaldehyde (DEAB; an inhibitor of retinoic acid synthases; 10 nM) in 96-well plates. The cells were harvested and examined for expression of CCR9, $\alpha_4\beta_7$, and FoxP3.

Total splenocytes (5 $\times$ 10^6; free of $FoxP3^+CD4^+$ cells) prepared from DO11.10 $rag2^{-/-}$ mice were adoptively transferred into BALB/c mice via a tail vein. The following day, the host mice were immunized s.c. with OVA (100 μg/mouse) in IFA. Every day, ATRA (10 mg/kg) was injected s.c. into the site of immunization. Seven days postimmunization, the mice were sacrificed and single-cell suspensions were prepared from indicated organs. The retinoid-induced $FoxP3^+$ cells in several tissue sites were identified with anti-DO11.10 TCR (KJ-1.26) and anti-FoxP3 Ab (FJK-16s).

The effect of retinoic acid on Th17 generation

The $CD4^+CD25^-CD44^-CD69^-$ T cells (2 $\times$ 10^5) were activated with coated anti-CD3 (2C-11, 5 μg/ml) and anti-CD28 Ab (37.51, 2 μg/ml) for

6 days in the presence of hIL-2 (50 U/ml), anti-IFN-γ (XMG2.4, 10 μg/ml), anti-IL-4 (11B11, 10 μg/ml), murine (m) IL-6 (2 ng/ml, PeproTech) and hTGF-β1 (5 ng/ml) with or without ATRA (10 nM). The cultured cells were activated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 h in the presence of monensin (10 μg/ml; Sigma-Aldrich) at 37°C. The cells were fixed and permeabilized for staining with PE-conjugated anti-mIL-17 (TC11-18H10.1; BioLegend) and analyzed with a FACSCalibur (BD Biosciences).

In vivo homing study

Retinoid-induced mouse FoxP3$^+$ T cells were prepared by culturing CD4$^+$CD25$^-$CD44$^-$CD69$^-$ T cells with Con A, IL-2 (50 U/ml), hTGF-β1 (5 ng/ml), and ATRA (10 nM) for 6 days. Control FoxP3$^+$ T cells were prepared by culturing the CD4$^+$CD25$^-$CD44$^-$CD69$^-$ T cells with Con A, IL-2 (50 U/ml), and hTGF-β1 (5 ng/ml) for 6 days. Control FoxP3$^+$ T cells were labeled with the red fluorescent dye tetramethylrhodamine-5-(and-6)-isothiocyanate (1 μg/ml), while retinoid-induced FoxP3$^+$ T cells were labeled with the green dye CFSE (1.4 μM). The two T cell subsets (10^7 cells/mouse) were coinjected into BALB/c mice via a tail vein. T cells were isolated from various tissues of BALB/c mice 20–24 h postinjection and were analyzed by flow cytometry. The lamina propria lymphocytes were isolated after removing the epithelial cells with 5 mM EDTA (five times), digestion (three times, 45 min each) with 2 mg/ml collagenase (type 3; Worthington) and 100 μg/ml DNase I (Worthington), filtration through a nylon mesh, and centrifuge in a 40/75% Percoll-gradient. The cells were stained with Abs to CD4 and FoxP3 (FJK-16s). Normalized homing indexes (HI) were calculated by a formula (HI = a/b), where a = (number of ATRA-treated cells that migrated into the tissue site)/(number of IL-2-treated cells that migrated into the tissue site), and b = (number of ATRA-treated cells in input)/(number of IL-2-treated cells in input).

Statistical analyses

The Student paired two-tailed t test was used. Values of $p \leq 0.05$ were considered significant. All error bars shown in this article are SEM.

Results

ATRA, a biologically active vitamin A metabolite, induces FoxP3 expression in T cells

FoxP3 is the master transcription factor and best marker for a major regulatory T cell lineage, defined frequently by expression of CD4 and CD25. We examined whether retinoic acid can induce FoxP3 expression in T cells. For this, human naive CD4$^+$CD25$^-$ T cells (>99.9% were FoxP3$^-$CD4$^+$ cells) were isolated from neonatal cord blood, which is a good source of Ag-inexperienced naive CD4$^+$ T cells. Naive CD4$^+$CD25$^-$ T cells were activated in the presence and absence of the vitamin A metabolite ATRA, and they were examined for expression of FoxP3 mRNA and protein (Fig. 1). When the FoxP3$^-$ T cells were cultured with ATRA, expression of FoxP3 mRNA was induced in the T cells at levels higher than the T cells cultured without ATRA (Fig. 1*A*). Also at the protein level, many more T cells cultured in the presence of ATRA than in its absence expressed FoxP3 (Fig. 1*B*). We will hereafter call these cells "retinoid-induced FoxP3$^+$ T cells." Expression of FoxP3 protein in retinoid-induced FoxP3$^+$ T cells was detected in the nuclei (Fig. 1*C*). The optimal ATRA concentration in induction of FoxP3 was only ~2 nM (Fig. 1*D*). The positive role of ATRA in increasing the numbers of FoxP3$^+$ T cells is clear also when absolute numbers of FoxP3$^+$ T cells are considered (Fig. 1*D*, *right*), suggesting that the enrichment of FoxP3$^+$ T cells is due to induction and proliferation of FoxP3$^+$ T cells but not preferential elimination of FoxP3$^-$ T cells by ATRA. In this regard, there was no difference in cell survival/death rates between control and ATRA-treated T cells at days 7 and 14 (data not shown).

We also examined the ATRA-induced expression of FoxP3 in T cells stimulated with different agonists such as Abs to CD3 and CD28 (Fig. 1*E*) or allogeneic DCs (Fig. 1*F*). Optimal induction of retinoid-induced FoxP3$^+$ T cells required activation of the co-stimulation receptor CD28 together with activation of CD3. When

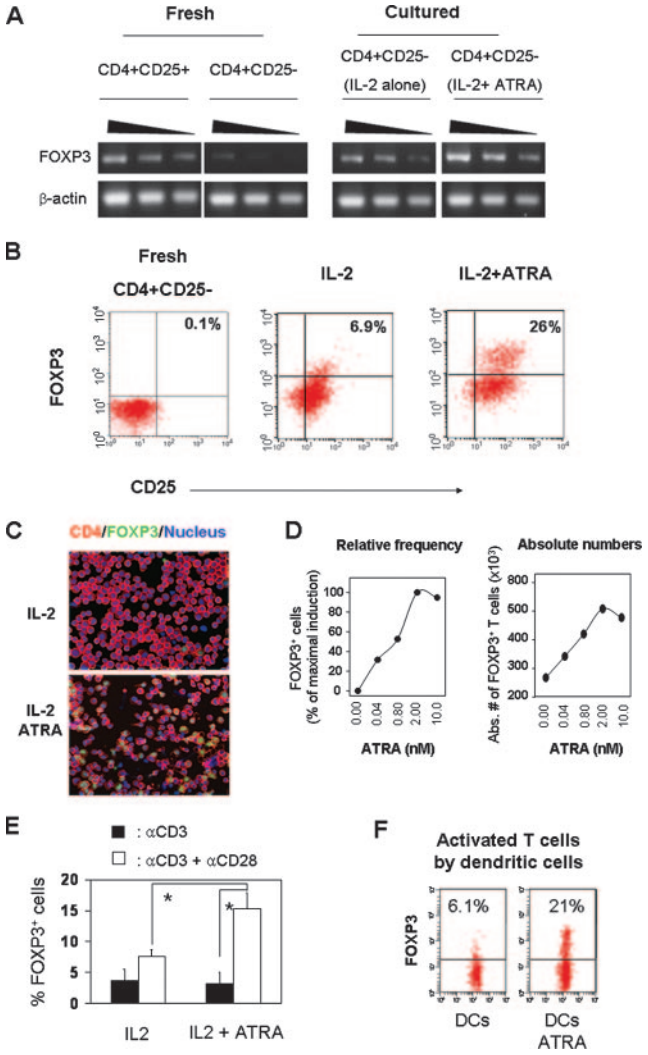

FIGURE 1. ATRA induces expression of FoxP3 in human T cells. *A*, Semiquantitative RT-PCR analysis of FoxP3 mRNA. (*B*) Flow cytometry and (*C*) cytospin-immunohistochemistry analyses of nuclear FoxP3 protein expression (original magnification ×200). *D*, Dose-dependent induction of intracellular FoxP3 protein by ATRA. *E*, ATRA enhances FoxP3 expression induced by activation of CD3 and CD28. *F*, ATRA enhances the generation of FoxP3$^+$ regulatory T cells by DCs. Cord blood CD4$^+$ CD25$^-$ naive T cells were cultured with PHA (*A–D*), indicated Abs (*E*), or with allogeneic monocyte-derived DCs at 50:1 (DC:T) ratio (*F*) for 6–7 days in the presence and absence of ATRA (2 nM) with IL-2 (25 U/ml). FoxP3 expression by activated (CD25$^+$) CD4$^+$ T cells is shown. Representative (*A–D* and *F*) or combined (*E*) data of three independent experiments are shown. *, Significant differences ($p < 0.05$).

stimulated by allogeneic monocyte-derived DCs, naive T cells became FoxP3$^+$ cells only at low efficiencies. Importantly, this process was considerably enhanced by ATRA (Fig. 1*F*).

Because IL-2 is a known inducer of FoxP3 (44), we examined the FoxP3 induction activity of ATRA together with high and low concentrations of IL-2. Induction of retinoid-induced FoxP3$^+$ T cells was clearly detected at both low (25 U/ml) and high (100 U/ml) concentrations of IL-2 (Fig. 2), but it was clearer at the low concentration of IL-2 due to the low background of FoxP3 expression. As reported by others (45), T cell activation was able to induce expression of FoxP3 in the presence of high IL-2 with the peak at day 3. When the absolute numbers of FoxP3$^+$ T cells were examined, many more FoxP3$^+$ T cells were accumulated in the presence of ATRA than in its absence over the 12-day period.

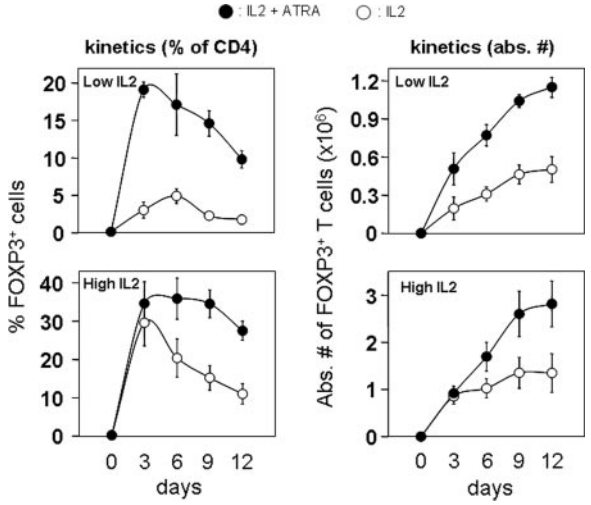

FIGURE 2. Effects of IL-2 on generation of retinoid-induced FoxP3$^+$ cells. Neonatal human cord blood CD4$^+$CD25$^-$ naive T cells were cultured in the presence and absence of ATRA in a T cell activation condition with IL-2 and PHA for 3 days and then in a resting condition for additional 3–9 days. High (100 U/ml) and low (25 U/ml) concentrations of IL-2 were used. Combined data of three independent experiments with means and SEM are shown. The data are shown in relative frequencies (*left panels*; percentage of CD4$^+$ T cells) and absolute numbers (*right panels*) of FoxP3$^+$ T cells.

Together with ATRA, the FoxP3 expression was maintained at higher levels even after day 12. Because of these results, 25 U/ml IL-2 was used to demonstrate the FoxP3 induction activity of ATRA (Figs. 3 and 4), and 100 U/ml IL-2 was used for the functional tests of the retinoid-induced FoxP3$^+$ T cells (Figs. 5–8) in this study. No FoxP3 induction was observed in the absence of IL-2 (data not shown).

Histone acetylation has been shown to be associated with increased gene transcription (46). The induction of both FoxP3 mRNA and protein by ATRA prompted us to examine the histone (H4) acetylation status of the FoxP3 promoter by a ChIP assay (Fig. 3). Histone acetylation of the proximal and distal regions of the *FoxP3* promoter was considerably more enhanced in ATRA-treated T cells than in control (IL-2 alone-treated) T cells. No increased histone acetylation by ATRA was observed in the *β-globin* gene.

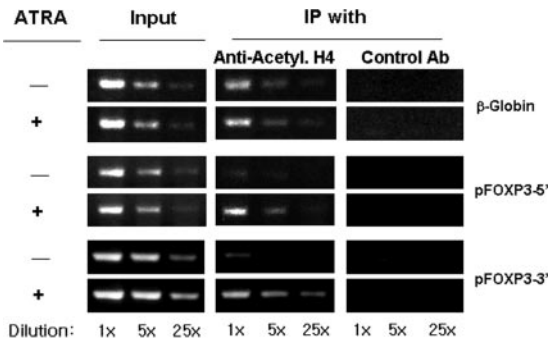

FIGURE 3. ATRA induces histone acetylation in the *FoxP3* promoter region. A ChIP assay was performed to determine histone acetylation in the *FoxP3* promoter. Cord blood CD4$^+$ CD25$^-$ naive T cells were cultured in the presence and absence of ATRA (2 nM) in a T cell activation condition with IL-2 (25 U/ml) and PHA for 3 days and then in a resting condition for additional 3–4 days. The *β-globin* gene was included as an internal control. Representative data from four independent experiments are shown.

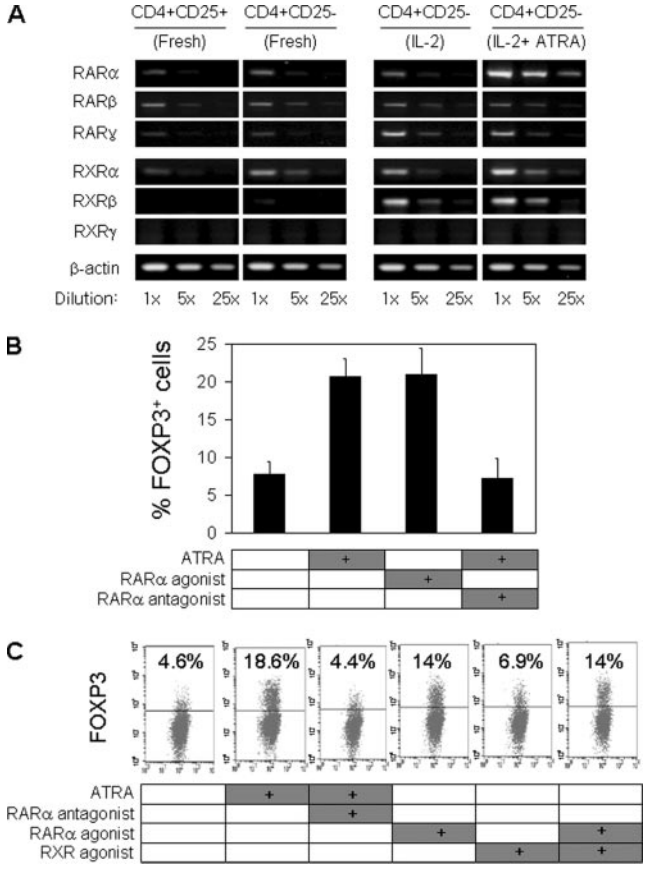

FIGURE 4. Role of RARα in FoxP3 induction by ATRA. *A*, Expression of retinoid receptors at mRNA level by freshly isolated and cultured T cells. RT-PCR was performed and a representative set of results of four independent experiments is shown. *B* and *C*, The RARα antagonist, Ro41-5253, completely suppressed the induction of retinoid-induced FoxP3$^+$ cells. Expression of the FoxP3 protein in CD4$^+$ T cells, determined by intracellular staining by anti-FoxP3 Ab, is shown as FACS dot plots. Neonatal human cord blood CD4$^+$CD25$^-$ T cells were cultured with IL-2 (25 U/ml) and PHA in the presence and absence of indicated agonists or antagonists. The concentrations of agonists or antagonists used in the experiment were 2 nM (ATRA), 1 μM (Ro41-5253), 5 nM (AM-580, RARα-specific agonist), and 20 μM (methoprenic acid, a pan-RXR agonist), all determined to be optimal in preliminary titration experiments.

Expression of RARs in ATRA-treated T cells

RARα, RARβ, and RARγ are the receptors for ATRA and, thus, are the likely receptors to mediate the signal to induce FoxP3. We determined mRNA expression of RARs in freshly isolated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells, and in the CD4$^+$CD25$^-$ T cells cultured in the presence and absence of ATRA (Fig. 4*A*). Expression of the three RARs along with RXRα and β was detected at low levels in freshly isolated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. Expression of RARγ and RXRβ was increased in IL-2-treated cells, and expression of RARα, RARγ, RXRα, and RXRβ was increased in ATRA-treated T cells. Among the receptors, expression of RARα was notably increased in the T cells cultured with ATRA (Fig. 4*A*).

ATRA induces FoxP3 expression in T cells through RARα

Because RARα is greatly induced in T cells in response to ATRA, we examined the function of this receptor in induction of FoxP3 using a RARα-specific antagonist, Ro41-5253. The RARα antagonist completely suppressed the induction of FoxP3 by ATRA (Fig. 4, *B* and *C*), suggesting that RARα is a dominant receptor

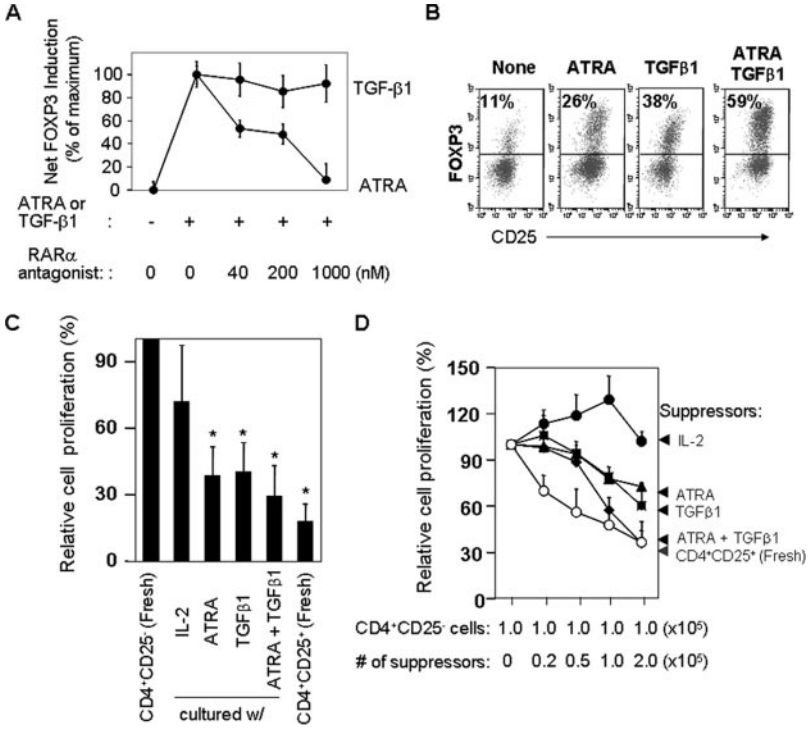

FIGURE 5. ATRA-cultured T cells are hypoproliferative and suppressive in function. *A*, Specific involvement of RARα in ATRA-mediated, but not in TGF-β1-mediated, induction of FoxP3. Ro41-5253 was used to block RARα. Combined data of three independent experiments are shown. *B*, Combined effects of ATRA and TGF-β1 in induction of FoxP3. Cultured T cells were examined for their proliferative potentials (*C*) or suppressive activities on proliferation of CD4$^+$CD25$^-$ cells (*D*). Neonatal human cord blood CD4$^+$CD25$^-$ naive T cells were cultured in the presence and absence of ATRA and/or TGF-β1 (2 ng/ml) with IL-2 (100 U/ml) and PHA for 3 days and then in a resting condition with IL-2 for an additional 3–4 days. Indicated cultured T cells were examined for their proliferative (10^5 cells/well, *C*) or suppressive activities on proliferation of freshly isolated CD4$^+$CD25$^-$ cells (10^5 cells/well) at various effector (i.e., cultured T cells) to target (i.e., CD4$^+$CD25$^-$ cells) ratios (*D*). Freshly isolated CD4$^+$CD25$^+$ T cells (FoxP3$^+$ cell content $\approx$90%) were also included as a control. Percent cpm levels relative to that of the target CD4$^+$CD25$^-$ cells alone (average cpm $\pm$ SEM was 30,301 $\pm$ 4,515) are shown. Combined data of three independent experiments with means and SEM are shown (*A*, *C*, and *D*). $*$, Significant differences from the IL-2 control ($p < 0.05$).

that mediates the effect of ATRA. We also used a RARα-specific agonist, AM-580. In a manner similar to ATRA, AM-580 was able to induce the FoxP3 protein in T cells (Fig. 4*B*). The optimal concentration of AM-580 for induction of FoxP3 was ~5 nM (titration not shown). A pan-RXR agonist, methoprenic acid, had only marginal effects on induction of FoxP3 at concentrations as high as 20 μM (Fig. 4*C*). The RARα and RXR agonists, when acted together, did not induce FoxP3 at higher than the levels induced by the RAR agonist alone. Taken together, these data support the role of RARα in ATRA-induced FoxP3 expression.

Both ATRA and TGF-β1 can induce FoxP3 expression, but the TGF-β1-mediated induction is RARα independent

Both ATRA and TGF-β1, when added separately to culture of CD4$^+$CD25$^-$ T cells, had clear effects on induction of FoxP3$^+$ T cells (Fig. 5, *A* and *B*). The RARα antagonist, Ro41-5253, blocked only ATRA-induced but not TGF-β1-induced FoxP3 induction (Fig. 5*A*), demonstrating the selective involvement of RARα in generation of ATRA-induced FoxP3$^+$ cells. When added together, TGF-β1 and ATRA did not have synergistic effects on the induction of human FoxP3 (Fig. 5*B*).

Effector functions of ATRA-treated T cells

Regulatory T cells are hypoproliferative and can suppress the proliferation of target cells. We assessed the proliferative and suppressive activities of ATRA-treated T cells. Similar to TGF-β1-treated T cells, ATRA-treated T cells had low proliferation potentials compared with freshly isolated CD4$^+$CD25$^-$ cells and IL-2-treated T cells (Fig. 5*C*). As expected, freshly isolated CD4$^+$CD25$^+$ cells, highly enriched with FoxP3$^+$ cells (~90%), were most hypoproliferative among the cell populations. ATRA-treated T cells suppressed the proliferation of CD4$^+$CD25$^-$ T cells at levels similar to that of TGF-β1-treated T cells. The T cells treated with both TGF-β1 and ATRA were even more suppressive (Fig. 5*D*). Overall, the suppressive activities were closely correlated with the FoxP3$^+$ cell content of the cell populations.

Regulatory T cells may use a number of different effector mechanisms to suppress target cells. Granzymes are a class of such effector molecules implicated in regulatory T cell function. We examined the expression of granzymes in retinoid-induced FoxP3$^+$ cells (Fig. 6). Although T cell activation in IL-2 weakly induced granzyme A, ATRA was highly efficient in induction of the molecule (Fig. 6, *A* and *B*). Interestingly, many retinoid FoxP3$^+$ cells expressed granzyme A, while the FoxP3$^+$ cells, induced with TGF-β1, did not express granzyme A at significant levels (Fig. 6, *A* and *B*). Freshly isolated FoxP3$^+$ cells and cultured CD4$^+$CD25$^+$ cells in the absence of ATRA also did not express granzymes. The high expression of granzyme A by retinoid-induced FoxP3$^+$ cells is unique in that activated CD8$^+$ T cells examined as a control highly expressed granzyme B but not granzyme A (Fig. 6*B*). Granzyme A was expressed within the granules of ATRA-treated T cells (Fig. 6*A*). The granzyme A expression in the FoxP3$^+$ T cells, induced by retinoic acid, was effectively suppressed by TGF-β1 (Fig. 6*C*). Because of their granzyme expression, we examined the cell-killing activity of ATRA-treated and control T cells (TGF-β1- or IL-2-treated T cells; Fig. 6*D*). ATRA-treated T cells were more efficient in killing target cells (activated B cells) than TGF-β1- or IL-2-treated T cells. This cell-killing activity was mostly abrogated by EGTA (a divalent cation chelator), which inhibits the perforin polymerization process required to deliver granzymes into target cells. Expression of granzyme B and perforin was somewhat increased in retinoid-induced FoxP3$^+$ T cells (Table I). In contrast, the retinoid FoxP3$^+$ cells did not express FasL, another cell killing molecule (Table I). No obvious difference in production of cytokines (IL-2, IL-4, IL-10, IL-13, IFN-γ, and TNF-α) was observed between vitamin A FoxP3$^+$ and IL-2-induced FoxP3$^+$ cells (Table I).

ATRA induces FoxP3$^+$ cells with homing receptors for intestine/mucosal tissues

Homing capacity is important for effector functions of T cell subsets in vivo. We next examined the homing receptor

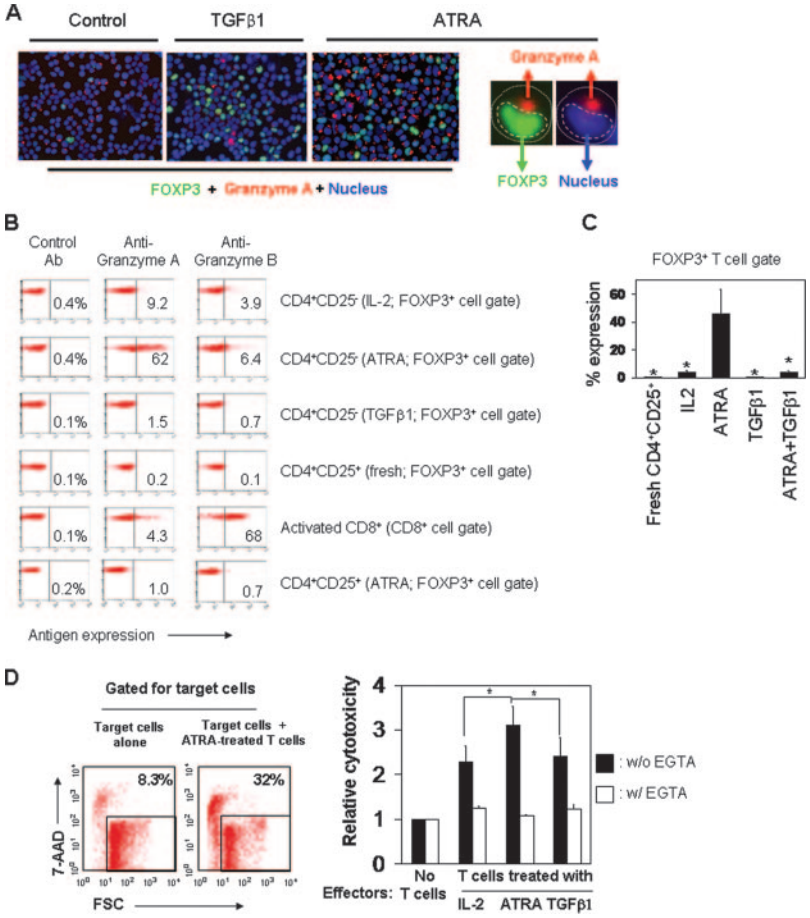

FIGURE 6. ATRA-treated T cells highly express granzymes and kill target cells. *A*, $CD4^+$ T cells, cultured with IL-2 (100 U/ml) alone, or IL-2 with ATRA (2 nM) or TGF-β1 (2 ng/ml), were examined for expression of granzyme A by immunofluorescence microscopy (original magnification $\times$200). *B*, $FoxP3^+$ T cells, generated from cord blood $CD4^+CD25^-$ T cells with IL-2 alone or together with ATRA or TGF-β1, were examined for their expression of granzyme A and granzyme B by flow cytometry analysis. Control cells such as freshly isolated and cultured $CD4^+CD25^+$ cells (cultured in IL-2 and PHA for 7 days) and activated $CD8^+$ T cells (activated CB $CD8^+$ T cells for 3 days with PHA) were included for comparison. *C*, TGF-β1 suppresses the expression of granzyme A in $FoxP3^+$ T cells. The data obtained from five independent experiments were combined, and averages and SEM are shown. *D*, Activated B cells, stained with CFSE, were cocultured with the suppressor cells for 4 h at 1:10 (target:effector) ratio, and killed target cells were detected based on 7-AAD staining and/or decreased forward scatter (FSC). The superantigen SEB was added to activate T cells, and EGTA was added to block the perforin-dependent cell-killing pathway. Representative (*A*, *B*, and *D*) or combined data of three independent experiments with means and SEM (*C* and *D*) are shown. *, Significant differences from the $FoxP3^+$ T cells induced with ATRA or between the two paired groups ($p < 0.05$).

phenotype of retinoid-induced $FoxP3^+$ cells (Fig. 7*A*). Retinoid-induced $FoxP3^+$ cells expressed a number of trafficking receptors: homeostatic secondary lymphoid (CCR7 and L-selectin), Ag-induced inflammatory (CCR4 and CXCR3, data not shown), and mucosal tissue-homing receptors (CCR9 and integrin β_7). Only the mucosal tissue-homing receptors CCR9 and integrin β_7 were uniquely expressed by the retinoid $FoxP3^+$ cells compared with control (IL-2-induced) $FoxP3^+$ cells. We observed similar induction of $\alpha_4\beta_7$ in $FoxP3^+$ cells by ATRA (data not shown). In contrast, the expression of CD62L was decreased on retinoid-induced $FoxP3^+$ cells compared with IL-2-induced $FoxP3^+$ cells. Retinoid-induced $FoxP3^+$ cells were highly responsive to the CCR9 ligand CCL25 (a chemokine specifically expressed by intestinal epithelial cells) in chemotaxis (Fig. 7*B*),

Table I. *The phenotype of ATRA-induced (i) FOXP3$^+$ cells differentiated from conventional naive CD4$^+$ T cells*[a]

	Molecules	IL2 iFoxP3$^+$	ATRA iFoxP3$^+$	TGF-β1 iFoxP3$^+$
Trafficking receptors	CCR9	3.2 ± 0.7	63.8 ± 0.9[b,c]	0.7 ± 0.1
	Integrin β_7	28.8 ± 0.7	64.2 ± 2.9[b,c]	17.5 ± 3.0
	CCR7	72.8 ± 0.6	71.4 ± 3.0[c]	97.9 ± 0.5
	L-selectin	87.9 ± 4.2	62.5 ± 12.5[b,c]	94.9 ± 1.9
	αE	11.5 ± 1.6	3.9 ± 0.1[b,c]	46.7 ± 5.5
Cytokines[d]	IL-2	12.1 ± 3.7	8.2 ± 2.2	11.7 ± 3.4
	IL-4	3.0 ± 0.5	2.7 ± 0.3[c]	0.6 ± 0.1
	IL-10	5.1 ± 1.1	3.9 ± 0.9[c]	5.8 ± 0.7
	IL-13	3.1 ± 0.2	3.5 ± 1.2	1.5 ± 0.2
	INF-γ	3.5 ± 0.8	1.9 ± 0.4[c]	0.4 ± 0.2
	TNF-α	73.5 ± 4.2	60.9 ± 5.6[c]	82.4 ± 3.1
Cytotoxic effector molecules	Granzyme A	9.7 ± 1.5	54 ± 5.3[b,c]	3.7 ± 0.6
	Granzyme B	2.5 ± 0.5	4.9 ± 0.6[b,c]	0.6 ± 0.2
	Perforin	0.8 ± 0.1	3.5 ± 1.3	0.7 ± 0.2
	FasL	1.3 ± 0.4	0.8 ± 0.5	1.2 ± 0.2

[a] Indicated human $FoxP3^+$ cells were generated as described in Fig. 5. Percent expression of the Ags by $FoxP3^+$ T cells is shown. Combined data (averages and SEM) of at least three independent experiments are shown.
[b] Significant differences from IL-2 iFoxP3$^+$ cells.
[c] Significant differences from TGF-β1 iFoxP3$^+$ cells.
[d] Intracellular cytokine expression was measured after activation with PMA and ionomycin.

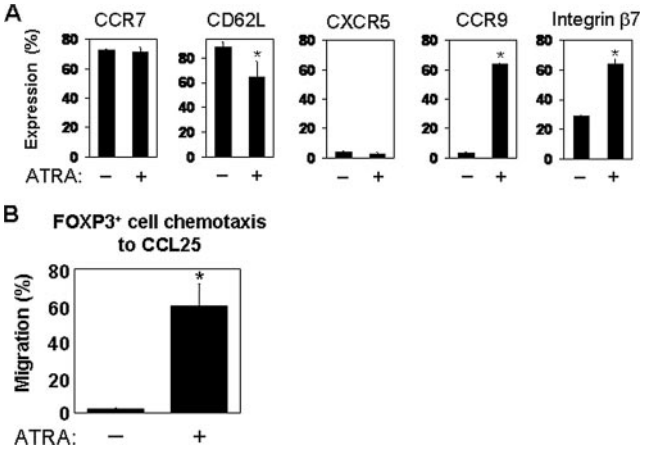

FIGURE 7. Retinoid-induced FoxP3$^+$ T cells express the mucosal tissue-homing receptors ($\alpha_4\beta_7$ and CCR9) and migrate to the intestinal epithelial cell-expressed chemokine CCL25. Neonatal human cord blood CD4$^+$CD25$^-$ T cells were cultured in a T cell activation condition (100 U/ml IL-2 and PHA) in the presence and absence of ATRA for 6 days. Cells were stained with mAbs to chemokine receptors or adhesion molecules for flow cytometry (*A*). Chemotaxis assay to CCL25 (2.5 μg/ml; an optimal concentration determined by preliminary titration experiments) was performed using a transwell migration system (*B*). Three independent experiments were combined, and averages and SEM are shown. $*$, Significant differences ($p < 0.05$).

demonstrating that CCR9, uniquely expressed by the retinoid FoxP3$^+$ cells, was fully functional.

ATRA promotes TGF-β1-induced generation of FoxP3$^+$ cells but suppresses TGF-β1 and IL-6-induced generation of Th17 cells

We next examined whether ATRA can induce FoxP3$^+$ cells in mouse T cells (Fig. 8*A*). We cultured the naive (CD4$^+$CD25$^-$CD44$^-$CD69$^-$) spleen T cells with ATRA in the presence of TGF-β1 at a suboptimal concentration (0.5 ng/ml) for 6–7 days in a T cell activation condition. ATRA reliably induced FoxP3$^+$ T cells only in the presence of TGF-β1. At high concentrations of TGF-β1 ($\geq$5 ng/ml), the effect of ATRA is small because TGF-β1 alone can induce FoxP3$^+$ cells at very high levels ($\sim$90%, data not shown). Both suppressive FoxP3$^+$ T cells and inflammatory Th17 cells can be induced by TGF-β1 (47, 48). Because ATRA enhanced the generation of TGF-β1-dependent mouse FoxP3$^+$ T cells, we examined whether ATRA can modulate the induction of Th17 cells as well (Fig. 8*B*). Surprisingly, ATRA considerably decreased the generation of Th17 cells.

Generation of retinoid-induced FoxP3$^+$ T cells by intestinal DCs and in vivo

We examined whether the DCs isolated from the intestine (combined CD11c$^+$ cells sorted from PP and small intestinal lamina propria) can convert the FoxP3$^-$ CD4$^+$ naive T cells into FoxP3$^+$ T cells. Splenic DCs were examined for comparison. The intestinal, but not spleen, CD11c$^+$ DCs were able to induce FoxP3$^+$ T cells at detectable levels. Most of the FoxP3$^+$ T cells expressed CCR9, and this induction was completely abolished by DEAB, an inhibitor of retinaldehyde dehydrogenases (retinoic acid synthases) that are expressed in these DCs (30).

To examine the generation of retinoid-induced FoxP3$^+$ T cells in vivo, we adoptively transferred DO11.10 rag2$^{-/-}$ splenocytes (containing FoxP3$^-$, but not FoxP3$^+$, CD4$^+$ cells) into BALB/c mice and immunized (s.c.) them with OVA. We injected the host mice with ATRA s.c. every day for 7 days. The reason that we used the s.c. route is to induce FoxP3$^+$ T cells in peripheral lymph nodes (PLN), which normally do not produce the gut-homing FoxP3$^+$ T cells, and thus it is easier to distinguish the ATRA-induced FoxP3$^+$ T cells from pre-existing gut-homing FoxP3$^+$ T cells. The ATRA injection induced $\alpha_4\beta_7{}^+$FoxP3$^+$ T cells, whereas the same immunization without ATRA did not induce this

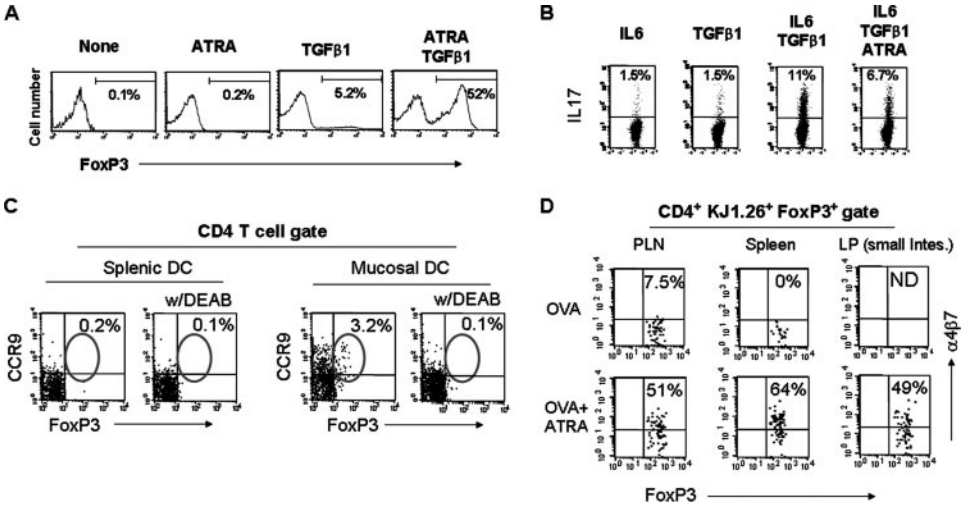

FIGURE 8. ATRA induces gut-homing receptor-expressing FoxP3$^+$ cells in mice. *A*, Naive mouse CD4$^+$ T cells (CD4$^+$CD25$^-$CD44$^-$CD69$^-$ T cells) were cultured in a T cell activation condition for 5 days with TGF-β1 (0.5 ng/ml) and/or ATRA (10 nM). The cells were examined for the FoxP3 expression. *B*, Naive CD4$^+$ T cells were activated with coated anti-CD3 and anti-CD28 Abs for 6 days in the presence of hIL-2, anti-IFN-γ, anti-IL-4 (10 μg/ml), mIL-6 (2 ng/ml), and hTGF-β1 (5 ng/ml) with or without ATRA (10 nM). The cells were examined for intracellular expression of IL-17. *C*, For induction of retinoid-induced FoxP3$^+$ T cells, CD11c$^+$ DCs were cocultured for 5 days with equal numbers of purified syngeneic CD4$^+$ naive (CD25$^-$CD44$^-$CD69$^-$) T cells in the presence of the superantigen SEB to provide a polyclonal Ag signal. After culture, the cells were examined for the expression of CCR9, $\alpha_4\beta_7$, and FoxP3. *D*, DO11.10 rag2$^{-/-}$ spleen cells that do not contain FoxP3$^+$ cells were adoptively transferred into BALB/c mice, and the host mice were immunized s.c. at the flank with OVA. Every day, ATRA (10 mg/kg) was injected s.c. into the site of immunization. Seven days postimmunization, the mice were sacrificed, and the frequency of retinoid-induced FoxP3$^+$ cells in the indicated tissue sites was examined. Representative data from at least three different experiments are shown.

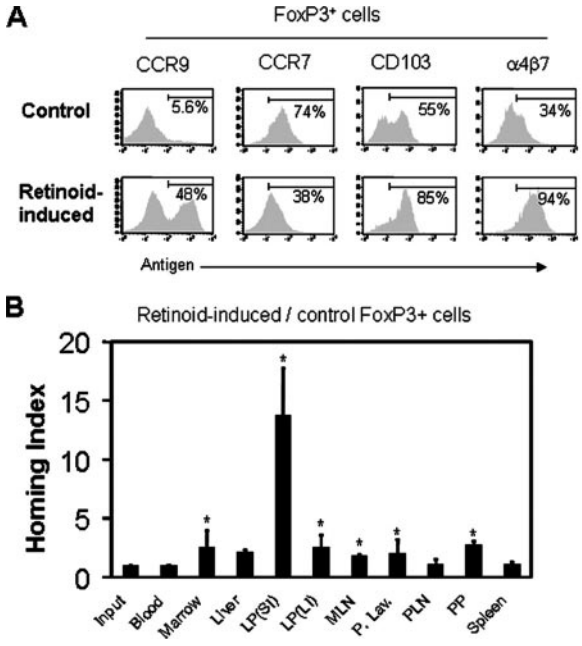

FIGURE 9. Retinoid-induced FoxP3$^+$ T cells express $\alpha_4\beta_7$, CCR9, and CD103 and preferentially migrate to the small intestine. *A*, Retinoid-induced mouse FoxP3$^+$ T cells (induced with TGF-β1 + ATRA) highly express $\alpha_4\beta_7$, CCR9, and CD103 compared with the control FoxP3$^+$ T cells induced by TGF-β1. *B*, Migration of the retinoid-induced vs control FoxP3$^+$ T cells to blood, marrow, liver, the lamina propria of small intestine (SI), the lamina propria of large intestine (LI), mesenteric lymph nodes (MLN), peritoneal lavage (P. Lav.), PLN, PP, and spleen. The relative homing indices were obtained by normalizing the numbers of retinoid-induced FoxP3$^+$ T cells with that of control FoxP3$^+$ T cells. The data from three different experiments were combined and averages and SEM are shown. *, Significant differences from the input ($p < 0.05$).

population in PLN and spleen (Fig. 8*D*). Converted FoxP3$^+$ T cells were found only in the small intestinal lamina propria of the mice injected with ATRA but not in the control mice (Fig. 8*D*), demonstrating the positive effect of ATRA on the generation of FoxP3$^+$ T cells in the gut.

The ATRA-induced mouse FoxP3$^+$ T cells specifically migrate to the gut and gut-associated secondary lymphoid tissues

We next examined the homing receptor phenotype of retinoid-induced mouse FoxP3$^+$ cells (Fig. 9*A*). Retinoid-induced mouse FoxP3$^+$ cells (induced from FoxP3$^-$ naive T cells by ATRA and TGF-β1) highly expressed mucosal tissue-homing receptors such as CCR9, $\alpha_4\beta_7$, and CD103 (the α-chain of $\alpha_E\beta_7$). In contrast, CCR7 expression was considerably down-regulated on the retinoid-induced FoxP3$^+$ T cells when compared with control FoxP3$^+$ T cells (induced by TGF-β1 alone). The control and retinoid-induced FoxP3$^+$ T cells were differentially labeled and injected i.v. into host mice to determine their homing capacity. Mice were sacrificed 20–24 h later, and the migration of the injected FoxP3$^+$ cells and FoxP3$^-$ T cells into 10 different tissue sites was determined. As shown in Fig. 9*B*, retinoid-induced FoxP3$^+$ T cells preferentially migrated to the small intestine. Migration of retinoid-induced FoxP3$^+$ T cells to other nonlymphoid tissue sites, PP, and mesenteric lymph nodes was somewhat increased as well.

Discussion

We investigated the function of retinoids in differentiation of naive CD4$^+$ T cells in vitro and in vivo using T cells isolated from human and mice. Our results revealed that retinoids can efficiently induce FoxP3 expression in T cells and generates a specialized FoxP3$^+$ regulatory T cell subset with a tissue tropism to the intestine and associated lymphoid tissues. One obvious difference between mouse and human FoxP3$^+$ T cells is that retinoic acid alone can induce the FoxP3 expression in human T cells but it additionally requires TGF-β1 (at suboptimal concentrations) to do the same thing in mouse T cells. Although retinoic acid enhances the generation of FoxP3$^+$ T cells, it suppresses the induction of Th17 cells by TGF-β1 and IL-6. Thus, retinoids can shift the T cell balance toward the mucosal tissue-homing FoxP3$^+$ T cells.

As retinoid nuclear receptors, RAR isotypes (α, β, and γ) and RXR isotypes (α, β, and γ) have been identified (49). Each receptor has multiple isoforms, generated through alternative mRNA splicing and/or transcription (50). ATRA preferentially binds RARs, whereas 9-*cis*-RA binds equally well to both RARs and RXRs (39). The two groups of retinoid nuclear receptors form RXR/RAR heterodimers, which can function as transcriptional activators upon interaction with RAR agonists (51). We found that ATRA induces FoxP3 in T cells. The optimal concentration range of ATRA for the induction in vitro is only 2–10 nM, which is considered a physiological concentration range (52). Freshly isolated naive T cells express RARα, β and γ at low levels. Among the RAR receptors, we found that the RARα message is greatly up-regulated in ATRA-treated T cells, suggesting a potential involvement of this receptor in induction of FoxP3. This positive role of RARα in induction of FoxP3 is supported by the fact that an RARα-specific antagonist completely blocked the generation of retinoid FoxP3$^+$ T cells.

T cell activation with agonistic anti-CD3 and CD28 Abs in the presence of IL-2 induces FoxP3 in human T cells (45). However, most of these FoxP3$^+$ T cells are not considered true regulatory T cells because the FoxP3 expression is transient, and these FoxP3$^+$ T cells do not have significant suppressive functions. Unlike these transient FoxP3$^+$ T cells, retinoid-induced FoxP3$^+$ T cells can maintain their FoxP3 expression and suppressive activities. We demonstrated also that the signals from CD28 activation and IL-2 can enhance generation of retinoid-induced human FoxP3$^+$ cells. More physiologically, ATRA can greatly enhance the generation of the FoxP3$^+$ T cells induced by DCs. We also found that intestinal DCs isolated from the mouse intestine can induce the FoxP3$^+$ T cells in a manner dependent on the retinoid synthesis pathway. These results suggest that retinoic acid, cytokines and the T cell activation signal provided by DCs can cooperatively induce the specialized FoxP3$^+$ T cells.

It is a matter of debate how FoxP3$^+$ T cells suppress target T cells. Regulatory T cells somehow inhibit stable contacts of naive T cells with APCs in mice (53). FoxP3$^+$ T cells express TGF-β1, CTLA4, and/or IL-10 (54, 55). FoxP3$^+$ T cells may suppress other T cells by producing IL-10 or expressing surface TGF-β1. In addition, it has been suggested that granzymes, CTLA4/indolamine 2,3-dioxygenase, and heme oxygenase-1 are potentially important effector molecules for regulatory T cells (56, 57). We observed that the naive human T cells cultured with ATRA were able to suppress the proliferation of CD4$^+$CD25$^-$ T cells. This suppressive activity of retinoid-induced human FoxP3$^+$ T cells per cell basis is thought to be as high as that of freshly isolated CD4$^+$CD25$^+$ T cells, given the fact that the frequencies of converted retinoid-induced FoxP3$^+$ T cells in the cultured T cells were only ~30%. We found that retinoid-treated human T cells highly express granzyme A, and they are more efficient in killing target cells than are TGF-β1-induced FoxP3$^+$ cells. We did not separate FoxP3$^+$ cells from FoxP3$^-$ T cells for the cell-killing assay because this requires nonsurvival intracellular staining. Therefore, the cell killing was probably mediated by not only granzyme A$^+$ FoxP3$^+$ cells but also

granzyme A$^+$ FoxP3$^-$ T cells. We also found that the granzyme A expression in the FoxP3$^+$ T cells, induced by retinoic acid, was effectively suppressed by TGF-β1 (Fig. 6*C*). Therefore, it is thought that the granzyme A expression in FoxP3$^+$ T cells is probably limited to human cells in certain conditions where no TGF-β1 is present. In line with this, mouse retinoid-induced FoxP3$^+$ T cells, induced with TGF-β1 and retinoic acid, did not express the granzyme A mRNA (unpublished data).

Recent studies suggest that FoxP3$^+$ T cells migrate to target tissue sites in a manner very similar to conventional T cells (8, 10, 11). Naive FoxP3$^+$ T cells, made in the thymus, express CCR7 and CD62L and migrate to secondary lymphoid tissues. Upon Ag priming, naive FoxP3$^+$ T cells acquire tissue-specific homing receptors to migrate to various nonlymphoid tissue sites. A difference between FoxP3$^+$ T cells and FoxP3$^-$ CD4$^+$ T cells is the fact that FoxP3$^+$ T cells more uniformly up-regulate a number of effector type trafficking receptors including CCR2, CCR4, CCR5, CCR6, CCR8, and CXCR3 (8, 10, 11). In a manner similar to conventional T cells, FoxP3$^+$ T cells also express, CD103, E-selectin, P-selectin, CCR7, CCR9, $\alpha_4\beta_7$, and CXCR5, the trafficking receptors important for migration into various tissue sites (2, 58–60). Among these, we found that retinoid-induced FoxP3$^+$ T cells uniquely express CCR9 and $\alpha_4\beta_7$. Additionally, the mouse FoxP3$^+$ T cells, induced with retinoic acid and TGF-β1, highly expressed CD103. These receptors are the major homing receptors for mucosal tissues, particularly, the intestine. Consistently, we demonstrated that ATRA-treated FoxP3$^+$ cells have a tissue tropism specific for the mucosal tissues particularly the small intestine.

In summary, we found that retinoids are effective converters of conventional naive FoxP3$^-$ T cells into a specialized FoxP3$^+$ T cell subset that we termed retinoid-induced FoxP3$^+$ T cells. This conversion of conventional T cells into regulatory T cells by retinoic acid is mediated through RARα and accompanies histone acetylation at the *FoxP3* promoter. Retinoid-induced FoxP3$^+$ T cells have a unique homing receptor phenotype and specifically migrate to the gut. Taken together, these results identify retinoids as positive regulatory factors for generation of gut-homing FoxP3$^+$ T cells.

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Disclosures

Some of the content of this manuscript is patent pending.

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