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Bile acids induce monocyte differentiation toward interleukin-12 hypo-producing dendritic cells via a TGR5-dependent pathway

Riko Ichikawa,[1] Tetsuro Takayama,[1] Kazuaki Yoneno,[1] Nobuhiko Kamada,[1] Mina T. Kitazume,[1] Hajime Higuchi,[1] Katsuyoshi Matsuoka,[1] Mitsuhiro Watanabe,[2] Hiroshi Itoh,[3] Takanori Kanai,[1] Tadakazu Hisamatsu[1] and Toshifumi Hibi[1]

[1]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, Keio University, Tokyo,* [2]*Division of Molecular Metabolism and System Medicine, School of Medicine, Keio University, Tokyo, and* [3]*Division of Endocrinology, Metabolism and Nephrology, Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan*

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Correspondence: T. Hibi and T. Hisamatsu, Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Emails: thibi@sc.itc.keio.ac.jp, hisamachi@a7.keio.jp
Senior author: Toshifumi Hibi

Summary

Dendritic cells (DCs) are known as antigen-presenting cells and play a central role in both innate and acquired immunity. Peripheral blood monocytes give rise to resident and recruited DCs in lymph nodes and non-lymphoid tissues. The ligands of nuclear hormone receptors can modulate DC differentiation and so influence various biological functions of DCs. The role of bile acids (BAs) as signalling molecules has recently become apparent, but the functional role of BAs in DC differentiation has not yet been elucidated. We show that DCs derived from human peripheral blood monocytes cultured with a BA produce lower levels of interleukin-12 (IL-12) and tumour necrosis factor-α in response to stimulation with commensal bacterial antigens. Stimulation through the nuclear receptor farnesoid X (FXR) did not affect the differentiation of DCs. However, DCs differentiated with the specific agonist for TGR5, a transmembrane BA receptor, showed an IL-12 hypo-producing phenotype. Expression of TGR5 could only be identified in monocytes and was rapidly down-regulated during monocyte differentiation to DCs. Stimulation with 8-bromoadenosine-cyclic AMP (8-Br-cAMP), which acts downstream of TGR5 signalling, also promoted differentiation into IL-12 hypo-producing DCs. These results indicate that BAs induce the differentiation of IL-12 hypo-producing DCs from monocytes via the TGR5-cAMP pathway.

Keywords: bile acid; cAMP; dendritic cell differentiation; interleukin-12; TGR5

Introduction

Dendritic cells (DCs) are classified as professional antigen-presenting cells and play a central role in both the innate and acquired immune responses. The DCs are a heterogeneous population of cells that can be divided into two major populations: (i) non-lymphoid tissue migratory and lymphoid tissue-resident DCs and (ii) plasmacytoid DCs. Migratory and resident DCs function in the maintenance of self-tolerance and the induction of specific immune responses against invading pathogens. The DCs act as antigen-presenting cells by phagocytosing pathogens and self antigens and then presenting the antigens on their cell surface to T and B cells. They also produce several cytokines in response to stimulation signals from pathogen-associated molecular patterns or

Abbreviations: ASBT, apical sodium-dependent bile salt transporter; BA, bile acid; BSEP, bile salt export pump; CBA, cytometric bead assay; CREB, cAMP response element binding protein; DC, dendritic cell; FXR, farnesoid X receptor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GUDCA, glycoursodeoxycholic acid; IL, interleukin; LCA, lithocholic acid; LPS, lipopolysaccharide; NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide C; qPCR, quantitative real-time polymerase chain reaction; TCDCA, taurochenodeoxycholic acid; TNF, tumour necrosis factor.

whole bacteria. Hence, DCs contribute to immunological homeostasis by promoting inflammatory responses to pathogens, inducing tolerance to self antigen, and suppressing excessive immune responses.[1,2] Dendritic cells play a critical role in the maintenance of immunological homeostasis and DC dysregulation can lead to autoimmune diseases and chronic inflammatory disorders. Abnormally excessive immune responses to commensal bacteria, food antigens and self antigens have been reported in the pathogenesis of these diseases. Therefore, conditioning DCs to display desirable properties, such as inducing an immunosuppressive DC phenotype, might represent a novel therapeutic strategy for these diseases. Recent studies have indicated that signalling through nuclear receptors, such as the retinoic acid receptor, the farnesoid X receptor (FXR) and the peroxisome proliferator-activated receptor-α, plays an important role in modulating the transcription of cytokine genes in innate immune cells.[3] Interleukin-1 (IL-12) produced by DCs has been implicated in promoting a type 1 helper T cell immune response and contributing to the pathogenesis of several chronic inflammatory disorders.[4–6] We previously demonstrated that Am80, a retinoic acid receptor agonist, promotes DC differentiation towards an IL-12 hypo-producing phenotype and that this molecule potentially represents a novel therapeutic molecule for inflammatory bowel disease.[7] The identification of similar molecules that induce an IL-12 hypo-producing DC phenotype might allow the development of novel therapeutic molecules for chronic inflammatory disorders. We hypothesized that bile acids (BAs), which are ligands for FXR and TGR5, might regulate DC differentiation and so we examined whether a BA can induce an IL-12 hypo-producing DC phenotype.

Bile acids are a family of steroid molecules generated in the liver by cholesterol oxidation. They accumulate in the blood, intestine and liver via enterohepatic circulation. In addition to their role in nutrient absorption, BAs are signalling molecules that can regulate immune cell responses via FXR and TGR5.[8] FXR is a member of the nuclear receptor superfamily of ligand-activated transcription factors[8–12] and is primarily expressed in enterohepatic tissues. FXR is known to regulate genes involved in BA synthesis, detoxification and excretion, and an increase in intracellular BA concentrations promotes transcriptional activation of FXR.[13–15] In addition, it has been reported that the FXR signalling pathway influences immunological responses such as cytokine production by immune cells.[16] TGR5 is a member of the rhodopsin-like superfamily of transmembrane G-protein coupled receptors that transduces signals through G proteins, and is activated by bile acids.[8,17]

In the present study, we show that BA treatment alters DC differentiation in a way that induces an IL-12 hypo-producing DC phenotype. Importantly, we found that the BAs affected DC differentiation through the TGR5-cAMP pathway, but not through FXR signalling. We found TGR5 to be expressed on the surface of monocytes, but not on differentiated DCs. Hence, our study demonstrates for the first time that BAs have the potential for modulating immune cell differentiation through the newly discovered transmembrane BA receptor, TGR5.

Materials and methods

Reagents

Recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from R&D Systems (Minneapolis, MN). Gel filtration grade lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich (St Louis, MO). Taurochenodeoxycholic acid (TCDCA) was purchased from Calbiochem (San Diego, CA). 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; Sigma-Aldrich) was kept as a 50 mM stock solution at −20° and diluted into complete medium immediately before use. The FXR agonist Fexaramine was purchased from Tocris Bioscience (Ellisville, MO). The TGR5-specific agonist [benzyl 2-keto-6methyl-4-(2-thienyl)-1,2,3,4-tetra-hydropyrimidine-5-carboxylate] was kindly provided by Dr Mitsuhiro Watanabe.[18]

Bacterial heat-killed antigen

The Gram-positive strain *Enterococcus faecalis* (ATCC29212) was cultured in brain–heart infusion medium. Bacteria were harvested and washed twice with ice-cold PBS. Bacterial suspensions were then heated at 80° for 30 min, washed, resuspended in PBS and stored at −80°. Complete killing was confirmed by 24-hr incubation at 37° on solid growth medium.

Dendritic cell culture

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood samples by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). The cells were aspirated from the gradient interface, washed in PBS and resuspended at 1×10^6 cells/ml in RPMI-1640 medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (BioSource, Camarillo, CA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, La Jolla, CA). Monocytes were purified using a magnetic cell separation system (MACS; Miltenyi Biotec, Auburn, CA) with anti-human CD14. Monocytes were seeded into six-well culture dishes at a density of 1×10^6 cells/well in 2 ml culture medium in the presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) to generate conventional immature DCs (cDCs).

Identical cultures were prepared with the bile acid TCDCA at the indicated concentrations for 6 days. We refer to cells cultured in these conditions as BA-DCs. We also investigated the effect of adding the BA to cultures on day 0, 2 or 4 together with GM-CSF/IL-4 treatment. In some experiments, monocytes were differentiated into DCs in the presence of GM-CSF and IL-4 with FXR agonist, TGR5 agonist and/or 8-Br-cAMP for 6 days.

Dendritic cells were stimulated with heat-killed *E. faecalis* (multiplicity of infection = 100) or LPS for 24 hr. Supernatants from stimulated DCs were collected and stored at −80° until cytokine assays were performed.

Cell viability assay

PrestoBlue Cell Viability Reagent (Invitrogen), diluted 1 : 10 with medium, was added to generated DCs (2×10^5 cells/100 μl diluted solution) in a 96-well plate. Samples were then incubated for 30 min at 37°. PrestoBlue is reduced from blue resazurin to red resorufin in the presence of viable cells. We then read the fluorescence (excitation 570 nm, emission 600 nm) with a Benchmark plus (Bio-Rad Laboratories Inc., Hercules, CA).

Cytokine measurement

The supernatants of DC cultures were measured for cytokine content by cytometric bead array (CBA) assays. A human inflammation CBA kit (BD Pharmingen, San Jose, CA) was used to quantify IL-12p70 and tumour necrosis factor-α (TNF-α) levels. Samples were analysed using a FACS Caliber flow cytometer (BD Pharmingen).

Flow cytometry

Cell surface marker fluorescence intensity was assessed using a FACS Caliber analyser and analysed using CellQuest (BD Pharmingen) or FlowJo (TreeStar Inc., Ashland, OR) software. Dead cells were excluded with propidium iodide staining. Monoclonal antibodies against CD14, CD80, CD83, CD86, CD40, CD1a, CD209 and CD205 were purchased from BD Pharmingen. Anti-TGR5 monoclonal antibody was purchased from R&D Systems.

Quantitative real-time PCR analysis

Total RNA was extracted from cells using an RNeasy Micro kit (Qiagen, Hilden, Germany), and cDNA was synthesized using a Quantitect RT kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and on-demand gene-specific primers, designed using the DNA Engine Opticon 2 System (Bio-Rad Laboratories, Inc.) and analysed with Opticon monitor software (MJ Research, Waltham, MA). The primers were as follows: *BSEP* (Hs00184824_m1), *NTCP* (Hs00161820_m1), *OATP* (Hs00366488_m1), *ASBT* (Hs01001557_m1), *TGR5* (Hs01937849_s1), *TNFα* (Hs00174128_m1), *IL-12p35* (Hs00168405_m1) and *IL-12p40* (Hs00233688_m1).

cAMP production assay

Monocytes (2×10^5 cells) were treated with lithocholic acid, TCDCA, glycoursodeoxycholic acid (GUDCA) and TGR5 agonist (5 μM) for 5 min in the presence of 1 mM 3-isobutyl-1-methylxanthine. The amount of cAMP was determined with a cAMP-Screen System (Applied Biosystems).

Phosphorylation of transcription factors

For intracellular phosphoprotein staining in monocytes we used a PhosFlow assay (BD Biosciences, Franklin Lakes, NJ). Cells in suspension were stimulated by TCDCA or with control medium for the indicated times, fixed with pre-warmed PhosFlow Cytofix solution for 10 min and permeabilized with ice-cold PhosFlow Perm buffer III for 30 min. Phycoerythrin-conjugated mouse anti-cAMP response element-binding protein (CREB) (pS133)/ATF-1 (pS63) or mouse anti-IgG isotype antibody was added to each tube and incubated at room temperature for 30 min in the dark. The cells were washed with 10 volumes of staining buffer and analysed by flow cytometry.

Statistical analysis

Statistical analysis was performed using GraphPadPrism software v. 4.0 (San Diego, CA). The statistical significance of differences between two groups was tested using a Student's *t*-test. For comparison of more than two groups, Kruskal–Wallis one-way analysis of variance (ANOVA) was used. If the ANOVA was significant, the Tukey–Kramer test was used as a post hoc test. Differences of $P < 0{\cdot}05$ were considered significant. All data are expressed as means ± SEM, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

Results

Morphology and expression of surface markers in BA-DCs, TGR5-DCs, cAMP-DCs and FXR-DCs

Conventional immature DCs were generated from monocytes by 6 days of culture with GM-CSF and IL-4. Other stimuli were added during the differentiation process; TCDCA (100 μM) for TCDCA-DCs, TGR5 agonist (20 μM) for TGR5-DCs, 8-Br-cAMP (10 μM) for cAMP-DCs, and fexaramine (100 μM) for FXR-DC. These DCs revealed different morphology and cell surface antigen

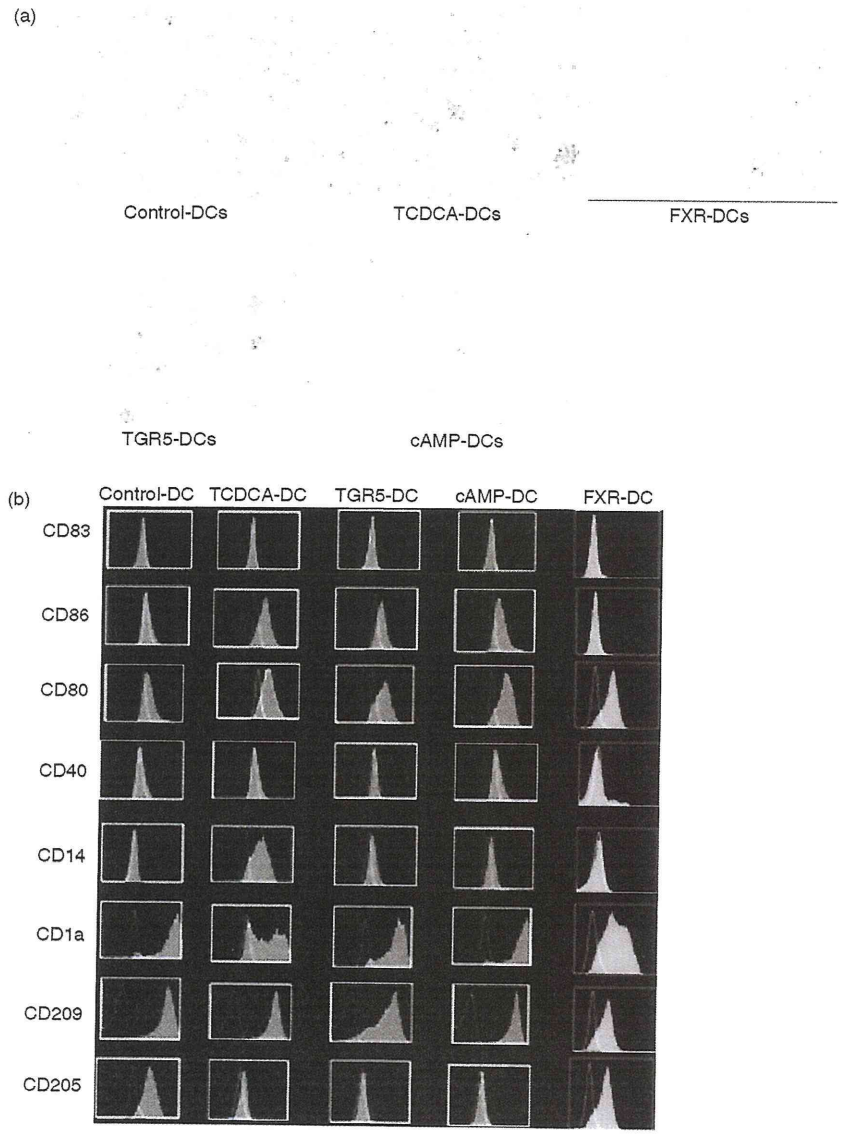

Figure 1. Morphology and expression of surface markers in bile acid-treated dendritic cells (BA-DCs), TGR5-DCs, cAMP-DCs and farnesoid X receptor (FXR)-DCs. (a) Conventional immature (cDCs) were generated from monocytes by 6 days of culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). Other stimuli were added during the differentiation process; taurochenodeoxycholic acid (TCDCA; 100 μM) for TCDCA-DCs, TGR5 agonist (20 μM) for TGR5-DCs, 8-Br-cAMP (10 μM) for cAMP-DCs and fexaramine (100 μM) for FXR-DC. (b) Differentiated DCs were harvested and the following surface markers and their isotype-matched monoclonal antibodies were analysed: CD83, CD86, CD80, CD40, CD14, CD1a, CD205 and CD209.

expression (Fig. 1a,b). We observed BA-DCs, TGR5-DCs and FXR-DC expressing low levels of CD1a, but not cAMP-DCs. Expression of co-stimulatory molecules, CD80 and CD86, was increased in BA-DCs, TGR5-DCs, cAMP-DCs and FXR-DCs. These findings demonstrated that TCDCA, TGR5 agonist, cAMP and FXR agonist induce different types of DCs during the 6-day differentiation culture. The viability of cDC, TCDCA-DCs, and TGR5-DCs was also confirmed (see Supplementary material, Fig. S1).

BA induces differentiation of IL-12 hypo-producing DCs in a dose-dependent manner

We have previously found that retinoic acid affects the differentiation of DCs from monocytes and induces anti-inflammatory DC differentiation.[7] We hypothesized that BAs might also affect the differentiation of DCs. To assess this, we cultured DCs differentiated from monocytes in the presence (referred to as BA-DCs) or absence (referred to as cDCs) of a BA and measured the cytokine-producing ability of these cells following stimulation with heat-killed antigen from the commensal bacteria *E. faecalis* or LPS + interferon-γ. The BA-DCs produced significantly less of the pro-inflammatory cytokines IL-12p70 and TNF-α in response to bacterial antigen or LPS + interferon-γ stimulation than cDCs, in a manner that was dependent on the concentration of the BA (Fig. 2a,b).

FXR agonist does not affect the differentiation of DCs from monocytes

We next investigated whether the FXR signalling pathway was involved in the DC differentiation process, using fexaramine, a powerful synthetic FXR agonist, in place of the BA during DC differentiation from monocytes.

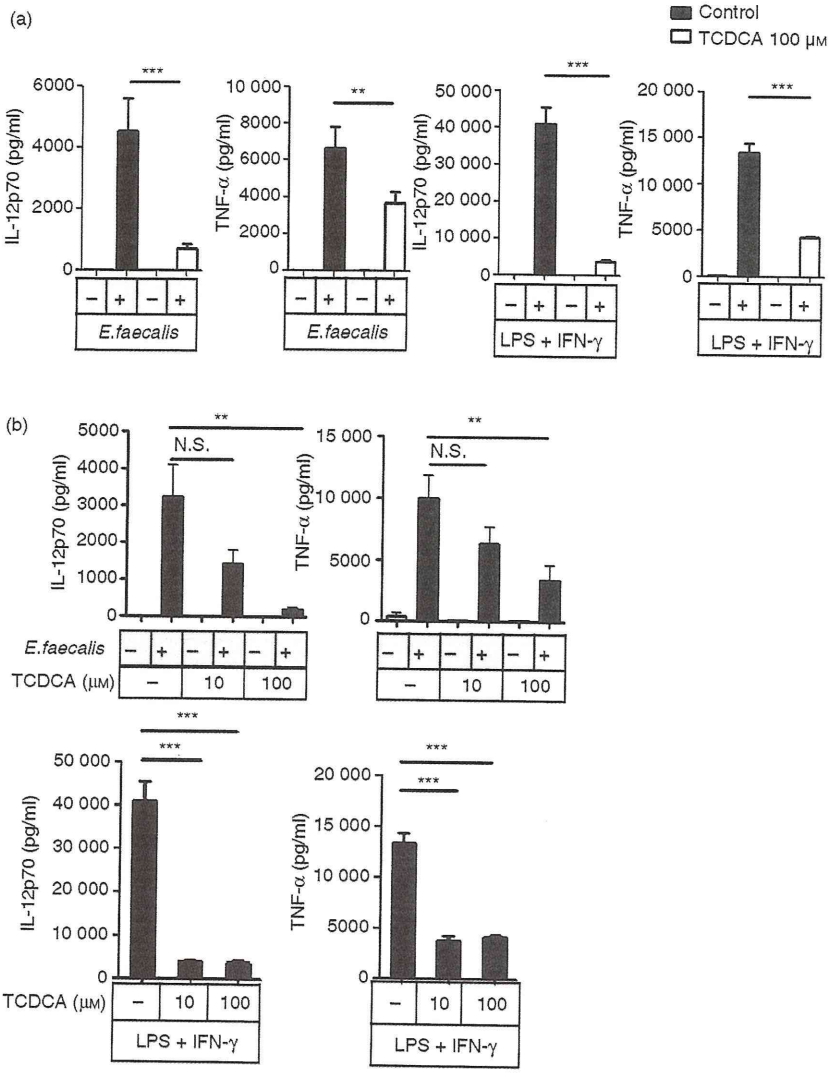

Figure 2. Bile acids (BAs) promote differentiation of interleukin-12 (IL-12) hypo-producing dendritic cells (DCs). (a) Human peripheral blood monocytes (1×10^6 cells/well in 2 ml culture medium) were cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF; 20 ng/ml) and IL-4 (20 ng/ml) with or without taurochenodeoxycholic acid (TCDCA; 100 μM). After 6 days in culture, DCs were stimulated with *Enterococcus faecalis* (multiplicity of infection = 100) or lipopolysaccharide (LPS; 100 ng/ml) + interferon-γ (IFN-γ; 100 ng/ml). The cytokines in culture supernatants were measured by cytometric bead array. Results show means ± SEM, control-DC, n = 12, TCDCA-DC, n = 12. (b) Monocytes were cultured as described above, with or without 100 μM or 10 μM TCDCA. IL-12p70 production in culture supernatants was measured by cytometric bead array. Results show means ± SEM, n = 6. Statistical analysis was performed by Kruskal–Wallis one-way analysis of variance and Tukey–Kramer test for multiple comparisons. Experiments were repeated at least three times.

Unexpectedly, DCs differentiated in the presence of the FXR agonist did not show the same IL-12 hypo-producing DC phenotype as DCs differentiated in the presence of the BA (Fig. 3a,b). We also examined mRNA expression of BA transporters, bile salt export pump (BSEP), organic anion transporting polypeptide C (OATP), sodium taurocholate cotransporting polypeptide (NTCP) and apical sodium-dependent bile salt transporter (ASBT) on monocytes and DCs. As shown in Fig. 3(c), no transporters for BAs were expressed on peripheral blood monocytes. The transporter BSEP was expressed in DCs, but all other transporters were absent in both monocytes and DCs.

IL-12 hypo-producing BA-DCs are induced through a TGR5 signalling pathway

As the FXR pathway did not appear to be involved in BA-DC differentiation, we next focused on TGR5. The DCs were differentiated from monocytes in the presence of a TGR5-specific agonist at several concentrations and IL-12 and TNF-α production in response to commensal bacterial antigen stimulation was measured. These TGR5-DCs produced less IL-12 and TNF-α than cDCs, in a similar manner to BA-DCs (Fig. 4a,b). We also measured the mRNA transcripts of TNF-α, IL-12p35 and IL-12p40 after stimulation with LPS and interferon-γ. We found that, at the mRNA level, expression of these pro-inflammatory cytokines was suppressed in TGR5-DCs (see Supplementary material, Fig. S2).

cAMP, a downstream target of TGR5, induces IL-12 hypo-producing DCs

We next assessed the mechanism by which BAs modify the differentiation of DCs to give an anti-inflammatory phenotype. It is known that cAMP has an immunosuppressive effect in various cells, so we measured cAMP levels of monocytes cultured with BA or the TGR5-specific agonist at several points during their differentiation to DC. Consistent with previous reports, the concentration

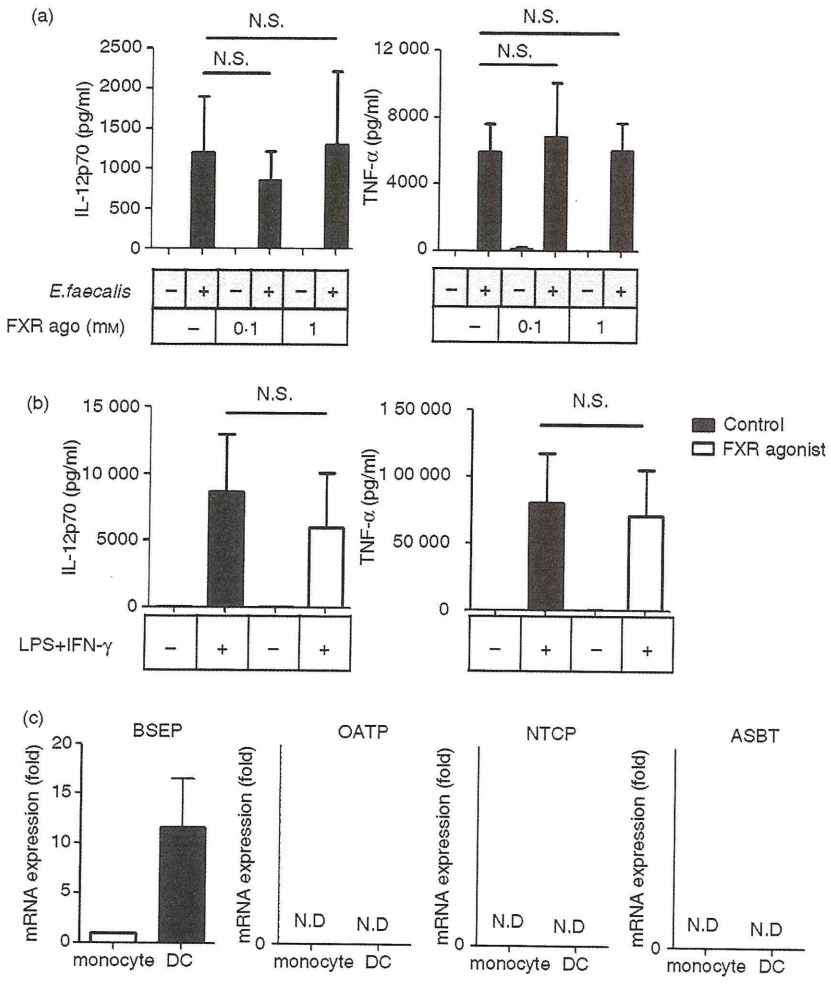

Figure 3. Effect of biles acids (Bas) on dendritic cell (DC) differentiation is independent of farnesoid X receptor (FXR). (a) DCs were differentiated from monocytes by treatment with granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and Fexaramine (100 μM or 1 mM) for 6 days (FXR-DC). Cytokine production after *Enterococcus faecalis* stimulation was measured. Results show means ± SEM, $n = 6$; differences were analysed by Kruskal–Wallis one-way analysis of variance. (b) FXR-DC (100 μM fexaramine) was stimulated with lipopolysaccharide (LPS; 100 ng/ml) and interferon-γ (IFN-γ; 100 ng/ml). Cytokine production was measured. Results show means ± SEM, $n = 6$; differences were analysed by Kruskal–Wallis one-way analysis of variance. Experiments were repeated at least three times. (c) No transporters for BAs are expressed on peripheral blood monocytes. We examined BA transporter expression in monocytes and DCs using real-time quantitative PCR. The transporter bile salt export pump (BSEP) was expressed in DCs, but all other transporters were absent in both monocytes and DCs. Statistical analysis was performed using paired *t*-tests $n = 3$.

of cAMP in monocytes increased following the administration of either BA or TGR5 agonist (Fig. 5a).[18] To test the hypothesis that this process induces anti-inflammatory DC differentiation, monocytes were treated with the cAMP analogue 8-Br-cAMP instead of the BA. The DCs obtained from this differentiation also produced lower levels of IL-12 and TNF-α than cDCs (Fig. 5b). Moreover, activation of CREB, a key molecule in cAMP downstream signalling,[8] was observed in monocytes treated with BA (Fig. 5c).

BA and TGR5 agonist can only influence the development of anti-inflammatory DCs from monocytes if present from the start of *in vitro* culture

Unexpectedly, the BA did not show any anti-inflammatory effect on terminally differentiated DCs (6 days after differentiation from monocyte) (Fig. 6a). To further investigate this discrepancy, we focused on the expression level of TGR5 on monocytes and DCs. We found TGR5 expression only in monocytes, and its expression was rapidly down-regulated over the course of differentiation to DCs, as assessed both by the surface expression of receptors and mRNA levels (Fig. 6b,c). Consistent with these results, the BA induced anti-inflammatory DCs when the BA was administrated on day 0, but not when the BA was added on day 2 or 4 after DC differentiation (Fig. 6d). Addition of the TGR5 agonist showed similar results (Fig. 6e). Next, we examined medium replacement experiments. As expected, DCs cultured in the presence of TGR5 agonist in the initial 3 days after DC differentiation (day 0–2) also showed an IL-12 hypo-producing phenotype (Fig. 6f).

Discussion

Both primary and secondary BAs can activate TGR5 and FXR, and several BAs have been reported to be natural ligands of TGR5. Of these lithocholic acid and taurolithocholic acid activate the TGR5 with an EC_{50} of ~ 600 and 300 nM, respectively, indicating that they can be considered physiological ligands for TGR5.[8,17,19–23] Other BAs activate TGR5 at micromolar concentrations. Chenodeoxycholic acid, which activates FXR at an EC_{50} of ~10 μM, is considered a physiological ligand for FXR. Other BAs can activate FXR at higher concentrations.[8–12]

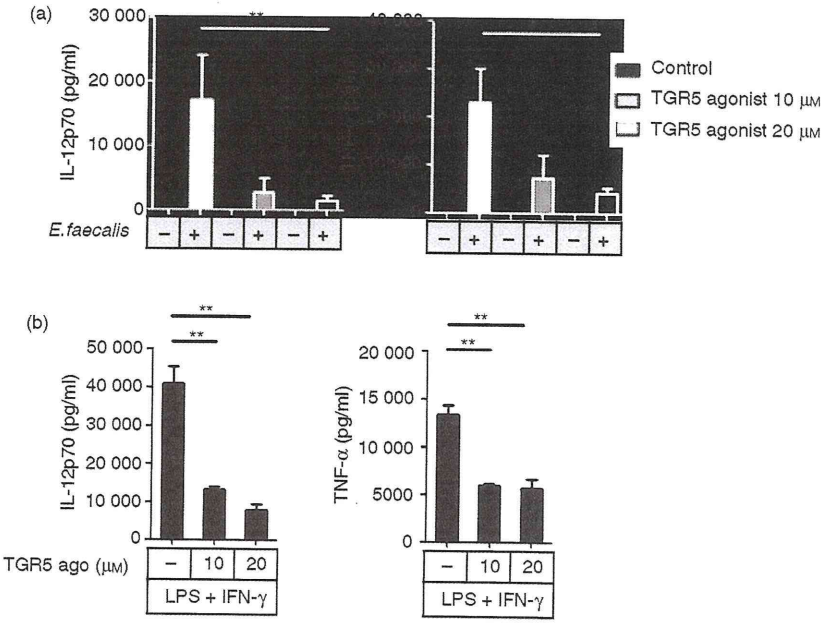

Figure 4. TGR5 agonist induces interleukin-12 (IL-12) hypo-producing dendritic cells (DCs). DCs were generated from monocytes by treatment with granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and TGR5 agonist (10 or 20 μM) for 6 days. Cytokine production in culture supernatants was analysed after 24-hr stimulation with *Enterococcus faecalis* (a) or lipopolysaccharide (LPS; 100 ng/ml) + interferon-γ (IFN-γ; 100 ng/ml) (b). Results show means ± SEM, TGR5 agonist 10 μM, $n = 4$, 20 μM, $n = 6$. Differences were analysed by Kruskal–Wallis one-way analysis of variance. Experiments were repeated at least three times.

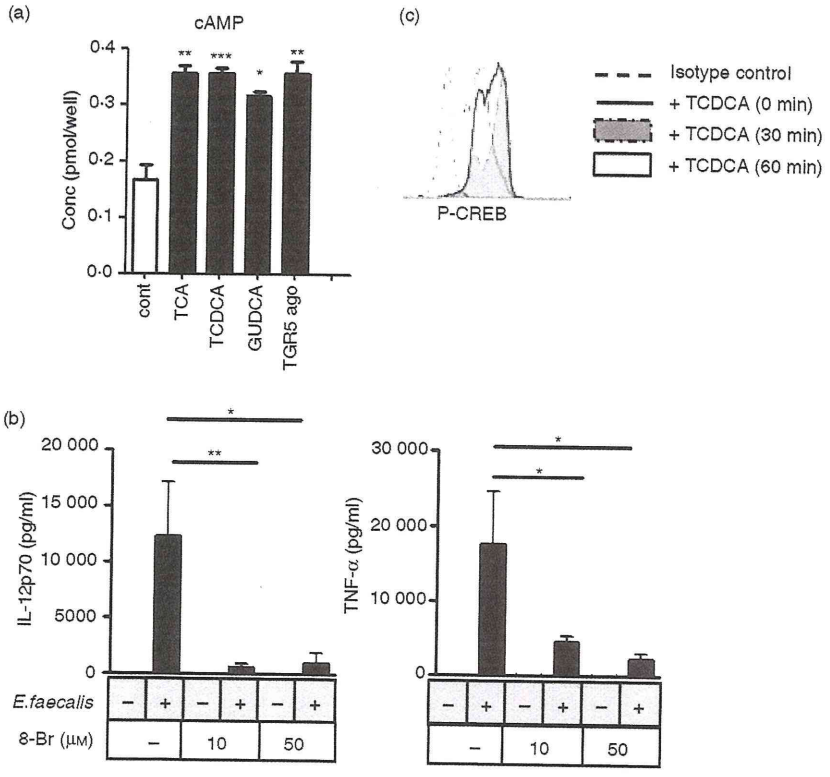

Figure 5. TGR5 signalling increases cAMP levels in monocytes, which induces differentiation into interleukin-12 (IL-12) hypo-producing dendritic cells (DCs). (a) Monocytes were treated with the indicated compounds at 5 μM (LCA, lithocholic acid; GUDCA, glycoursodeoxycholic acid; TCDCA, taurochenodeoxycholic acid). Results show means ± SEM, $n = 3$. (b) cAMP induces IL-12 hypo-producing DCs in a concentration-dependent manner. DCs were generated from monocytes with granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and 8-Br-cAMP (10 and 50 μM). After 6 days in culture, cytokine levels in culture supernatants were analysed after 24-hr stimulation with *Enterococcus faecalis*. Results show means ± SEM, $n = 6$. Data were analysed by Kruskal–Wallis one-way analysis of variance and Tukey–Kramer test for multiple comparisons. (c) Bile acid activates cAMP response element binding protein (CREB) in monocytes. Cells in suspension were stimulated by TCDCA (100 μM) or with control medium for 30 or 60 min, treated for PhosFlow analysis, and analysed using anti-CREB (pS133)/ATF-1 (pS63) or mouse IgG isotype antibodies for flow cytometry. Experiments were repeated at least three times.

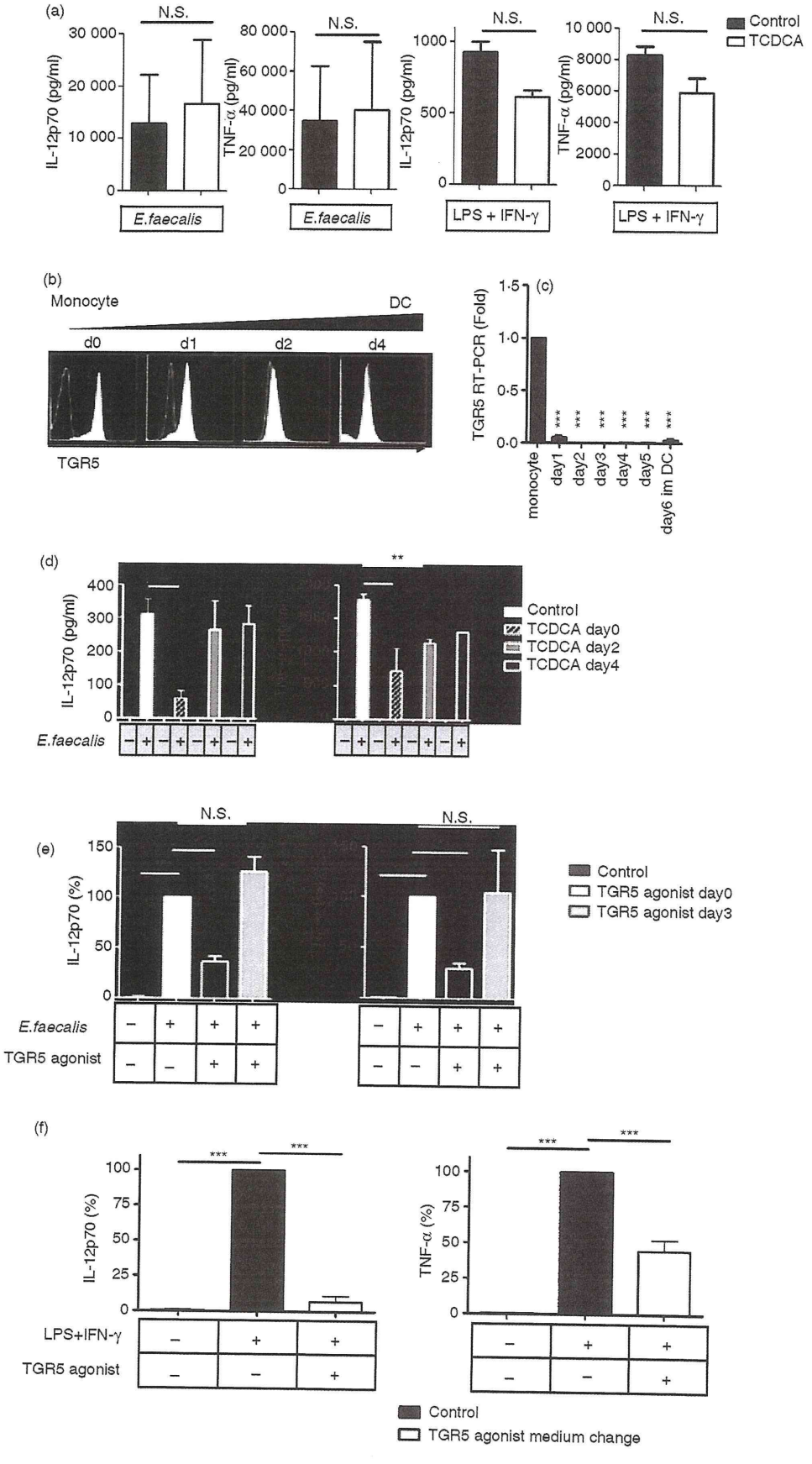

Figure 6. Bile acids (BA) do not directly suppress cytokine production from human dendritic cells (DCs). (a) After monocytes were cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF; 20 ng/ml) and interleukin-4 (IL-4; 20 ng/ml) for 6 days, DCs (5×10^5 cells/ml) were stimulated with *Enterococcus faecalis* (100 ng/ml) and lipopolysaccharide (LPS; 100 ng/ml) with or without bile acids (taurochenodeoxycholic acid; TCDCA; 100 μM). Cytokines in culture supernatants were measured by cytometric bead assay (CBA). Results show means ± SEM, $n = 5$. (b) Monocytes were differentiated into DCs by treatment with GM-CSF and IL-4 for 6 days and surface expression levels of TGR5 and its isotype-matched monoclonal antibodies on monocytes were analysed by flow cytometry on day 0, day 1, day 2 and day 4. (c) mRNA transcripts of TGR5 in $CD14^+$ monocytes and DCs were measured by quantitative PCR. The transcript level of TGR5 was normalized to the level of β-actin transcript. Results show means ± SEM of fold induction of at least four individual experiments. (d) DCs were generated from monocytes with GM-CSF and IL-4. TCDCA (50 μM) was added on day 0, day 2 and day 4. Cytokine levels in culture supernatants on day 6 were measured by CBA after 24-hr stimulation with *E. faecalis*. Results show means ± SEM, $n = 3$. (e) DCs were generated from monocytes with GM-CSF, IL-4. TGR5 agonist (20 μM) was added on day 0 and day 3. Cytokine levels in culture supernatants on day 6 were measured by CBA after 24-hr stimulation with *E. faecalis*. Amount of IL-12p70 and tumour necrosis factor-α (TNF-α) in culture supernatant were indicated as relative percentage of control DCs with stimulation. Results show means ± SEM, $n = 5$. $^{***}P < 0.001$. (f) DCs were generated from monocytes with GM-CSF and IL-4 (day 0–6). TGR5 agonist (20 μM) was added only on day 0–2. Cytokine levels in culture supernatants on day 6 were measured by CBA after 24 hr of stimulation with LPS + interferon-γ (IFN-γ). Amounts of IL-12p70 and TNF-α in culture supernatant were indicated as relative percentage of control DCs with stimulation. Results show means ± SEM, $n = 4$. $^{***}P < 0.001$. Data were analysed by Kruskal–Wallis one-way analysis of variance and Tukey–Kramer test for multiple comparisons.

We used 10–100 μM of TCDCA, a concentration range at which it can activate both FXR and TGR5.

TGR5 is expressed in several tissues, with the highest levels detected in the gall bladder, followed by the ileum and colon. TGR5 expression is not detectable in primary hepatocytes.[8,19] In contrast, FXR is highly expressed in the liver, intestine, kidney and adrenal glands.[8–10,13,24–27] FXR expression in immune cells, such as $CD14^+$ monocytes, has also been reported, but its expression in these cells is relatively low compared with the expression of other nuclear receptors such as LXRα (Liver X Receptor alpha).[3] In addition, we could not detect expression of BA transporter mRNA in monocytes. These findings are consistent with our demonstration that the FXR agonist did not influence DC differentiation in our experiments. In the present study, we found expression of TGR5 on $CD14^+$ peripheral blood monocytes. Furthermore, the presence of the TGR5-specific agonist promoted the differentiation of IL-12 hypo-producing DC in a similar manner to that seen in the presence of BA. Taken together, these results suggest that BAs can regulate the DC differentiation process through TGR5 expressed on primary peripheral blood monocytes.

Expression of TGR5 was rapidly down-regulated during DC differentiation from monocytes, and differentiated DCs did not express detectable levels of cell surface TGR5. Although the mechanisms of TGR5 gene transcription regulation have not been identified, our study of mRNA transcription revealed that the amount of TGR5 mRNA transcript was dramatically reduced following GM-CSF and IL-4 stimulation. In addition, it has been reported that ligand stimulation causes cellular internalization of TGR5.[8] These findings suggest that the binding of the BA to TGR5 on monocytes at the initial phase of differentiation is crucial if differentiation outcomes are to be influenced by the BA.

Activation of TGR5 leads to intracellular cAMP accumulation, which activates CREB.[8,18] The CREB then transactivates target genes by binding to the cAMP response element in the promoter region of these genes.[8,20,22,23] In our studies, stimulation of monocytes by BA or a TGR5-specific agonist led to up-regulated intracellular cAMP concentrations. It has been reported that intracellular cAMP concentration is an important modulator of pro-inflammatory cytokine transcription.[28] Consistent with these observations, treatment of monocytes with cAMP also promoted cellular differentiation into IL-12 hypo-producing DC. The cAMP promotes the differentiation of $CD14^+$ monocytes into $CD1a^{low}$ $CD209^+$ DCs.[29] We observed BA-DCs and TGR5-DCs, but not cAMP-DCs, expressing low levels of CD1a (Fig. 1), although all three DC types displayed a similarly low capacity to produce IL-12. Interestingly, FXR-DCs also showed a CD1a-positive DC phenotype, but FXR-DCs did not display an IL-12 hypo-producing phenotype.

The finding that BAs can induce the differentiation of IL-12 hypo-producing DCs through activation of the TGR5-cAMP pathway suggests that the TGR5 pathway may be a novel therapeutic target for T helper type 1 dominant chronic inflammatory disorders, such as Crohn's disease and psoriasis.[7,28,30] As BAs are part of the enterohepatic circulation, the ileum, mesenteric lymph node and liver may be candidates as sites where BAs act to modulate DC differentiation.

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Disclosure

The authors declare no conflict of interests.

Author contributions

RI, TT, KY performed the experiments. RI, TT, KY, NK, MK, HH, SO, MW, TK and HI designed the experiments, collected data and wrote the manuscript. T. Hisamatsu reviewed the manuscript and T. Hisamatsu and T. Hibi supervised and compiled the final version of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cell viability of peripheral blood monocytes-derived dendritic cells.

Figure S2. Messenger RNA transcript of pro-inflammatory cytokines in TGR5-dendritic cells.

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Regulatory T Cells Suppress Development of Colitis, Blocking Differentiation of T-Helper 17 Into Alternative T-Helper 1 Cells

TOMOHISA SUJINO,* TAKANORI KANAI,* YUICHI ONO,* YOHEI MIKAMI,* ATSUSHI HAYASHI,* TOMOMITSU DOI,* KATSUYOSHI MATSUOKA,* TADAKAZU HISAMATSU,* HIROMASA TAKAISHI,* HARUHIKO OGATA,* AKIHIKO YOSHIMURA,‡ DAN R. LITTMAN,§ and TOSHIFUMI HIBI*

*Division of Gastroenterology and Hepatology, Department of Internal Medicine and ‡Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan; §Molecular Pathogenesis Program, The Kimmel Center for Biology and Medicine of the Skirball Institute, New York University School of Medicine, New York, New York

See editorial on page 801.

BACKGROUND & AIMS: Although T-helper (Th) 17 and Th1 cells are involved in pathogenesis of intestinal inflammation, their developmental pathways and sufficiency to promote disease are not known; nor are the roles of $CD4^+CD25^+$ regulatory T (T_R) cells in their development. **METHODS:** We performed adoptive transfer experiments to investigate the induction and suppression of colitis using naïve $CD4^+CD45RB^{high}$ T cells and/or $CD4^+CD25^+$ T_R cells that were obtained from retinoid-related orphan receptor gamma t (RORγt) *gfp/*$^+$ or Ly5.1/Ly5.2 congenic mice. **RESULTS:** We observed 3 types of colitogenic $CD4^+$ Th1 cells (interleukin [IL]-17A$^-$interferon [IFN]-γ$^+$): RORγt$^-$ classical Th1 cells that differentiated directly from naïve T cells; RORγt$^+$ Th1-like cells; and RORγt$^-$ alternative Th1 cells that were terminally differentiated from RORγt$^+$ cells via Th17 (IL-17A$^+$IFN-γ$^-$), Th17/Th1 (IL-17A$^+$IFN-γ$^+$), or Th1-like (IL-17A$^-$IFN-γ$^+$) cells. In this pathway, $CD4^+CD25^+$ T_R cells suppress the development of not only classical Th1 cells, but also alternative Th1 cells at the transition of Th17/Th1 into alternative Th1 cells, resulting in accumulation of Th17 and Th17/Th1 cells in mice in which the development of colitis was suppressed. Furthermore, T_R cells regulated the established balance of Th17 and Th1 cells under colitic conditions to yield a high ratio of Th17 and Th17/Th1 cells to Th1 cells in noncolitic conditions. **CONCLUSIONS: Th17 and Th17/Th1 cells become colitogenic alternative Th1 cells via Th17, Th17/Th1, and Th1-like cells, independently of classical Th1 cells. T_R cells suppress this pathway, resulting in accumulation of Th17 and Th17/Th1 cells.**

Keywords: Inflammatory Bowel Disease; Mouse Model; T-Cell Development; Immune Response.

T helper-17 (Th17) cells are characterized by production of Th17 cytokines, such as interleukin (IL)-17A[1]; expression of retinoid-related orphan receptor gamma t (ROR γt)[2,3]; and induction of massive tissue inflammation in various immune diseases in a manner similar to T-bet transcription factor–governed IFN-γ–producing Th1 cells.[1,2,4] Interestingly, naturally occurring Th17 cells reside preferentially in the intestine in healthy mice,[3,5] and may control a variety of bacterial and fungal infections at mucosal sites.

Questions about the distinction and correlation between the roles of colitogenic Th17 and Th1 cells, and their pathogenicity in inflammatory bowel disease (IBD) remain largely unanswered. For instance, it has been reported that recombination activating gene (RAG)-2$^{-/-}$ mice transferred with naïve T cells obtained from either T-bet$^{-/-}$[6] or RORγ$^{-/-}$ mice[7] do not develop colitis. Furthermore, in animal IBD models,[8,9] a distinct subset of IL-17A$^+$IFN-γ$^+$ Th17/Th1 cells may participate in the pathogenesis of each disease. This is complicated further by a recent report showing that colitogenic "Th1-like" cells emerge directly from Th17 cells at a late stage of the colitis developmental process.[10]

Based on such complex backgrounds, we aimed to clarify not only the developmental pathway of Th17, Th17/Th1, and Th1 cells in the pathogenesis of colitis, but also the role of $CD4^+CD25^+Foxp3^+$ regulatory T (T_R) cells in their developmental pathway using an in vivo adoptive T-cell transfer model, in which RAG-1, 2$^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells develop chronic colitis 4–6 weeks after transfer.[11] This model is advantageous, as the transferred cells can be traced during development or suppression of colitis over time, without having to continually supply naïve $CD4^+$ T cells.[12] This model is controlled by T_R cells possibly by suppressing the generation and maintenance of colitogenic Th1 and Th17 cells.[7,13,14]

Materials and Methods

Mice

C57BL/6 (Ly5.1), C57BL/6 (Ly5.2), and C57BL/6-background RAG-2$^{-/-}$ (Ly5.2) mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice with a green fluores-

Abbreviations used in this paper: CTLA, cytotoxic T lymphocyte–associated antigen; GFP, green fluorescent protein; Gr, Group; IBD, inflammatory bowel disease; IFN, interferon; LP, lamina propria; mRNA, messenger RNA; RAG, recombination activating gene; RORγt, retinoid-related orphan receptor gamma t; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; T_R, regulatory T.

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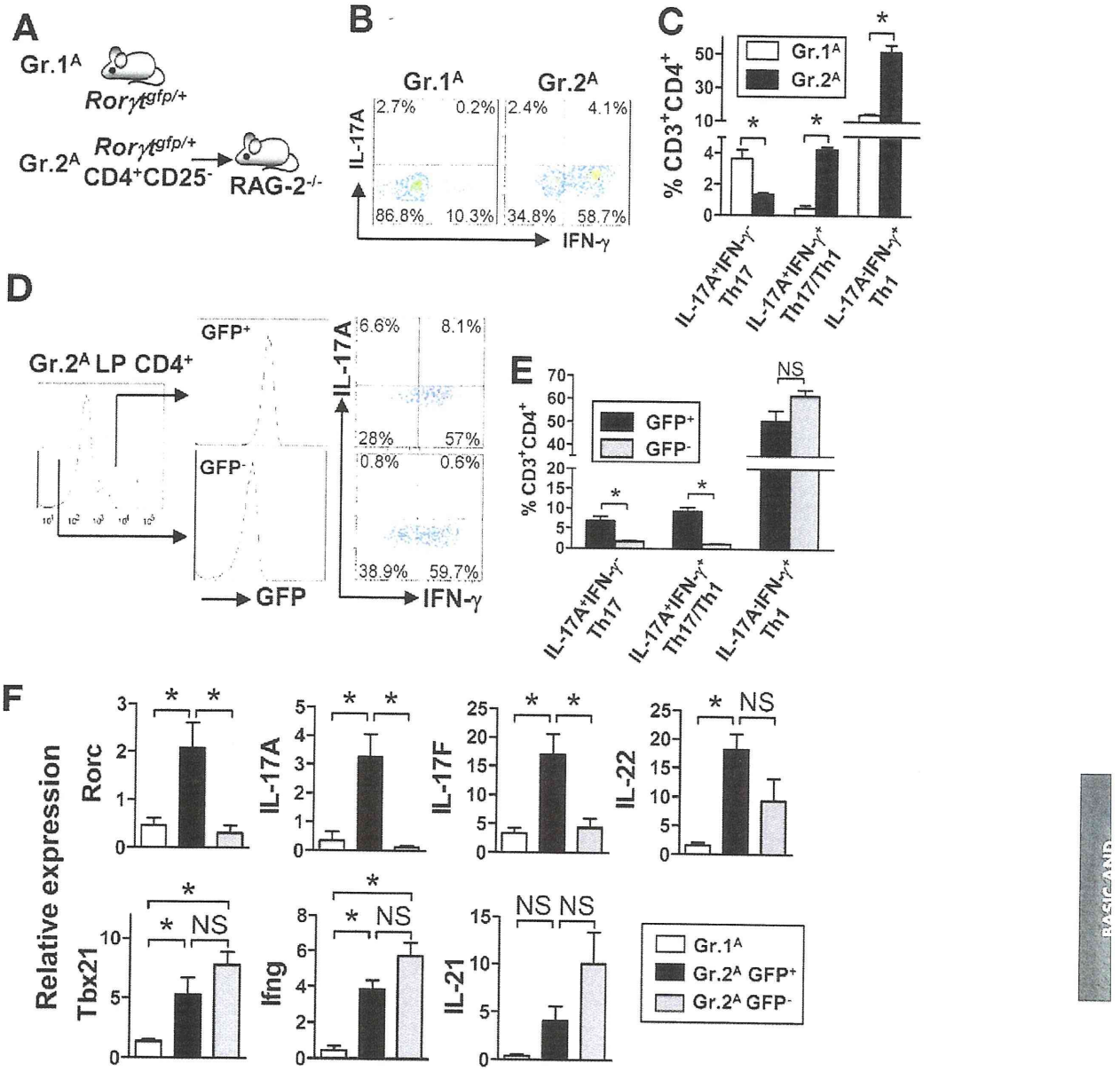


Figure 1. RORγt+ Th1-like cells and RORγt− Th1 cells co-reside in inflamed mucosa of colitis. (*A*) Transfer protocol A. RAG-2$^{-/-}$ mice were transferred with RORγt$^{gfp/+}$ CD4+CD25− T cells (Gr.2^{A}, n = 6). Age-matched RORγt$^{gfp/+}$ mice were used as a negative control (Gr.1^{A}, n = 6). Mice were sacrificed 6 weeks after transfer. (*B*) Expression of IL-17A and IFN-γ in colonic CD3+CD4+ T cells. (*C*) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in colonic LP CD3+CD4+ T cells. (*D*) Expression of IL-17A and IFN-γ in colonic GFP+ and GFP− CD3+CD4+ T cells of Gr.2^{A} mice. (*E*) Mean percentages of colonic Th17, Th17/Th1, and Th1 cells in the GFP+ and GFP− CD3+CD4+ T cells of Gr.2^{A} mice. (*F*) Expression of the indicated mRNA in the colonic CD3+CD4+ cells, normalized to Act-b expression. Statistical data (*C, E, F*) show mean ± standard error of mean (n = 6/group). *$P < .05$. NS, not significant.

cent protein (GFP) reporter complementary DNA knocked-in at the site for initiation of RORγt translation on the C57BL/6 (Ly5.2) background were described previously.[15] Mice were maintained under specific pathogen-free conditions. All experiments were approved by the regional animal study committees. (See Supplementary Materials and Methods for full details).

Results

RORγt+ Th1-Like and RORγt− Th1 Cells Co-Reside in the Inflamed Colitis Mucosa

We first assessed the presence of Th17 and Th1 cells in the inflammatory conditions of colitis following adoptive transfer of CD4+CD25− T cells obtained from RORγt$^{gfp/+}$ mice into RAG-2$^{-/-}$ mice (Group 2 in protocol A [Gr.2^{A}]) (Figure 1*A*). We confirmed that the transferred splenic CD4+CD25− T cells did not express GFP (data not shown). As expected, Gr.2^{A} mice developed severe colitis as assessed by weight loss (Supplementary Figure 1*A*), gross colon appearance (Supplementary Figure 1*B*), clinical score (Supplementary Figure 1*C*), histology (Supplementary Figure 1*D*), and the absolute number of colonic lamina propia (LP) CD4+ T cells (Supplementary Figure 1*E*), in contrast to age-matched RORγt$^{gfp/+}$ mice (Gr.1^{A}). The percentages of Th1 (IL-17A−IFN-γ+) and Th17/Th1

(IL-17A$^+$IFN-γ^+) cells in Gr.2^A mice were significantly higher than those in Gr.1^A, while the percentage of Th17 (IL-17A$^+$IFN-γ^-) cells in Gr.2^A mice was lower than that in Gr.1^A mice (Figure 1*B* and *C*). Notably, significant numbers of Th17/Th1 cells were only found in colitic Gr.2^A mice, but not in RORγt$^{gfp/+}$ Gr.1^A mice (Figure 1*B* and *C*). To further investigate the distinct differences between LP RORγt$^+$ and RORγt$^-$ CD3$^+$CD4$^+$ T cells in Gr.2^A mice with colitis, GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ cells were sorted (Figure 1*D*), and approximately 10% of LP CD3$^+$CD4$^+$ T cells in colitic Gr.2^A mice were found to be GFP-positive. As expected, almost no LP GFP$^-$CD3$^+$ CD4$^+$ cells in Gr.2^A mice expressed IL-17A, while half of them expressed IFN-γ. Surprisingly, however, almost half of the GFP$^+$CD3$^+$CD4$^+$ T cells expressed IFN-γ without retaining IL-17A expression, while a small but substantial percentage of those cells expressed IL-17A as Th17 (IL-17A$^+$IFN-γ^-) and Th17/Th1 (IL-17A$^+$IFN-γ^+) cells (Figure 1*D*). Although the percentages of Th17 and Th17/Th1 cells in GFP$^+$CD3$^+$CD4$^+$ T cells were small, they were significantly higher than those in GFP$^-$CD3$^+$CD4$^+$ T cells, while the percentage of dominant Th1 cells in GFP$^+$CD3$^+$CD4$^+$ T cells was comparable to that in GFP$^-$CD3$^+$CD4$^+$ T cells (Figure 1*E*). Control GFP$^+$ CD3$^+$CD4$^+$ T cells obtained from the colon and small intestine of normal RORγt$^{gfp/+}$ Gr.1A mice did not express IFN-γ, while GFP$^-$ CD3$^+$CD4$^+$ T cells obtained from the colon and small intestine of Gr.1A mice express significant levels of IFN-γ (Supplementary Figure 1*F*). Additionally, in vitro–stimulated (transforming growth factor [TGF]$-\beta$ + IL-6) GFP$^+$ Th17 cells expressed IL-17A, but not IFN-γ (Supplementary Figure 1*G*).

To further characterize GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells obtained from colitic Gr.2^A mice, we compared Th1 and Th17 marker messenger RNA (mRNA) expression. As expected, the expression levels of Th17 markers, such as Rorc, IL-17A, and IL-17F, in GFP$^+$CD3$^+$CD4$^+$ T cells in Gr.2^A mice were significantly higher than in GFP$^-$CD3$^+$CD4$^+$ T cells in Gr.2^A mice or CD3$^+$CD4$^+$ T cells in Gr.1^A mice, while expression of IL-22 and IL-21 in GFP$^+$CD3$^+$CD4$^+$ T cells tended to be reduced and increased when compared to that in GFP$^-$CD3$^+$CD4$^+$ T cells, respectively, although not significantly (Figure 1*F*). This suggests that IL-22 and IL-21 are not solely produced by Th17 cells, but possibly also by Th22, Th1, and T_{FH} cells, and not by NK-22 (CD3$^-$) cells. Surprisingly, however, GFP$^+$ T cells also expressed Th1 markers, such as Tbx21 (gene for T-bet)[10] and IFN-γ to a comparable level as GFP$^-$ cells (Figure 1*F*), suggesting that a portion of RORγt$^+$ CD3$^+$CD4$^+$ T cells are able to express T-bet, and produce IFN-γ but not IL-17A.

RORγt$^+$T-bet$^+$ Th1-Like Cells and RORγt$^-$T-bet$^+$ Th1 Cells in Colitic Mice Are Colitogenic

Given the possibility that there are at least 2 types of IFN-γ–expressing cells, Th1 (RORγt$^-$) and Th1-like (RORγt$^+$), in the inflamed mucosa of colitic mice, we asked whether each population is colitogenic after re-transfer into RAG-2$^{-/-}$ mice. To this end, new RAG-2$^{-/-}$ mice were re-transferred with LP GFP$^+$ (Gr.1^B) or GFP$^-$ (Gr.2^B) CD3$^+$CD4$^+$ T cells isolated from colitic RAG-2$^{-/-}$ mice previously transferred with CD4$^+$CD25$^-$ T cells obtained from RORγt$^{gfp/+}$ mice (Gr.2^A mice [Figure 1*A*]) (Figure 2*A*). Both groups of mice similarly developed colitis as assessed by weight loss (Supplementary Figure 2*A*), colon appearance (Supplementary Figure 2*B*), clinical score (Supplementary Figure 2*C*), histological score (Supplementary Figure 2*D*), and absolute number of infiltrating LP CD3$^+$CD4$^+$ T cells (Supplementary Figure 2*E*). Although GFP expression was retained in approximately half of the LP CD3$^+$CD4$^+$ T cells in Gr.1^B mice, but not induced in those cells in Gr.2^B mice (Figure 2*B* and *C*), the ratio of IL-17A$^-$IFN-γ^+ Th1 cells was comparable in the 2 groups (Figure 2*D* and *E*). In contrast, the ratio of Th17 and Th17/Th1 cells in Gr.1^B mice was significantly higher than that in Gr.2^B mice (Figure 2*D* and *E*). Notably, almost all GFP$^+$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice expressed T-bet, because the histogram showed 1 peak shift compared to the isotype control (Figure 2*F*, *upper*). Therefore, these RORγt$^+$T-bet$^+$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice were composed of 3 subpopulations; IL-17A$^+$IFN-γ^+ Th17/Th1 cells, IL-17A$^-$IFN-γ^+ Th1-like cells, and IL-17A$^-$IFN-γ^- double-negative cells (Figure 2*F*, *lower*), while almost all GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice expressed T-bet, and these RORγt$^-$T-bet$^+$ CD3$^+$CD4$^+$ T cells were composed of 2 subpopulations; Th1 cells and double-negative cells (Figure 2*F*). Like GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice, almost all GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.2^B mice expressed T-bet, and were composed of 2 subpopulations; Th1 cells and double-negative cells (Figure 2*F*). As we could not detect GFP expression in cells in Gr.2^B mice during this experiment, these cells included classical Th1 cells and "alternative Th1 cells" via RORγt$^+$ CD3$^+$CD4$^+$ T cells.

T_R Cells Not Only Suppress Development of Colitis, But Also Induce Accumulation of Th17 and Th17/Th1 Cells

We next investigated whether T_R cells affect the developmental pathway of Th17, Th17/Th1, Th1-like, and Th1 cells (Figure 3*A*). We confirmed that RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells (Ly5.1$^+$) and CD4$^+$CD25$^+$ T_R cells (Ly5.2$^+$) (Gr.2^C) did not develop colitis, as assessed by weight loss (Supplementary Figure 3*A*), colon appearance (Supplementary Figure 3*B*), clinical score (Supplementary Figure 3*C*), histological appearance and its score (Supplementary Figure 3*D*), and the absolute number of LP CD3$^+$CD4$^+$ T cells (Supplementary Figure 3*E*), in contrast to mice transferred with CD4$^+$CD45RB^{high} T cells alone (Gr.1^C), which developed colitis. Surprisingly, however, the percentages of not only Th17 cells, but also Th17/Th1 cells derived from the Ly5.1$^+$CD4$^+$CD45RB^{high} T-cell population in Gr.2^C mice were significantly higher than those in Gr.1^C mice, while the percentage of Th1 cells in Gr.2^C mice was significantly

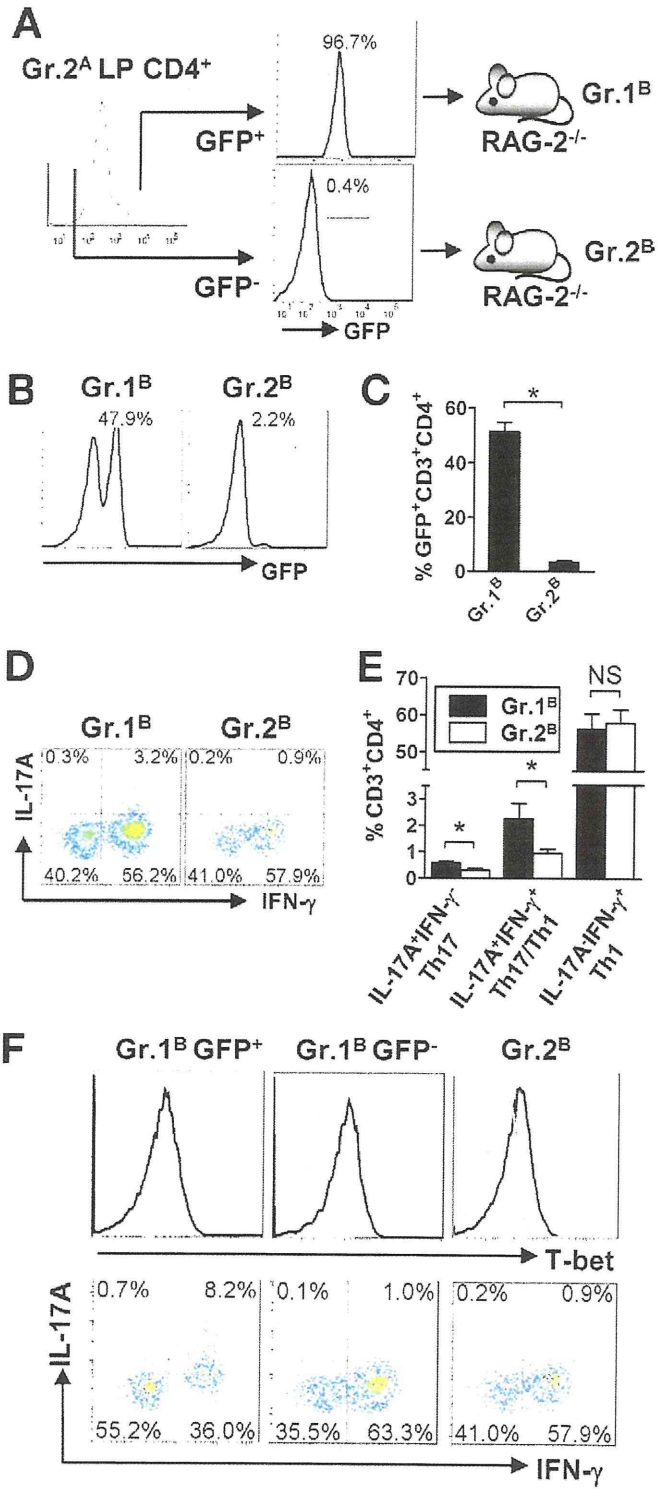


Figure 2. RORγt+T-bet+ Th1-like cells and RORγt−T-bet+ Th1 cells in colitic mice are colitogenic. (*A*) Transfer protocol B. RAG-2−/− mice were transferred with GFP+ ($Gr.1^B$, n = 6) or GFP− ($Gr.2^B$, n = 6) CD3+CD4+ T cells obtained from colitic $Gr.2^A$ mice in transfer protocol A (see Figure 1*A*). Mice were sacrificed 10 weeks after transfer. (*B*) Expression of GFP in colonic CD3+CD4+ T cells. (*C*) Percentage of GFP+ cells in colonic CD3+CD4+ T cells. (*D*) Expression of IL-17A and IFN-γ in colonic CD3+CD4+ T cells. Data are representative of six mice in each group. (*E*) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in the colonic CD3+CD4+ T cells. (*F*) Expression of T-bet and IL-17A/IFN-γ in GFP+ and GFP− CD3+CD4+ T cells (T-bet, black; Isotype, gray). Statistical data (*C*, *E*) show mean ± standard error of mean (n = 6/group). *$P < .05$. NS, not significant.

lower than that in $Gr.1^C$ mice (Figure 3*B-a* and *b*). We further assessed the expression of tumor necrosis factor (TNF)−α and cytotoxic T lymphocyte−associated antigen (CTLA-4) molecules, because TNF-α is one of the representative pathological molecules for the pathogenesis of IBD,[17] and CTLA-4 is a member of the inhibitory CD28 family, which are preferentially expressed in cells that have regulatory function, such as T_R cells.[18] Like expression pattern of IFN-γ, the percentages of Ly5.1-derived IL-17A+TNF-α− and IL-17A+TNF-α+ cells in noncolitic $Gr.2^C$ mice were significantly higher than those in $Gr.1^C$ mice, while the percentage of IL-17A−TNF-α+ cells in noncolitic $Gr.2^C$ mice was significantly lower than that in $Gr.1^C$ mice (Figure 3*C-a* and *b*). In contrast, the percentages of all 3 IL-17A+CTLA-4−, IL-17A+CTLA-4+, and IL-17A−CTLA-4+ subpopulations in noncolitic $Gr.2^C$ mice were significantly higher than those in $Gr.1^C$ mice (Figure 3*D-a* and *b*). Real-time quantitative polymerase chain reaction analysis confirmed that expression levels of Rorc, IL-17A, IL-17F, and IL-22 in Ly5.1+CD4+CD45RBhigh T-cell−derived CD3+CD4+ T cells in $Gr.2^C$ mice were significantly higher than those in $Gr.1^C$ mice, whereas those of Tbx21 and IFN-γ in Ly5.1+CD3+CD4+ T cells in $Gr.2^C$ mice were significantly lower than those cells in $Gr.1^C$ mice (Figure 3*E*). In contrast, Foxp3 mRNA was solely expressed in Ly5.2-derived T_R cells in $Gr.2^C$ mice, but not in Ly5.1+CD3+CD4+ T cells (Figure 3*E*).

T_R Cells Suppress Development of Colitis With the Increase of RORγt+ Th17 and Th17/Th1 Cells

To precisely determine whether T_R cells suppress the differentiation pathway of Th17, Th17/Th1, Th1-like, and Th1 cells in mice in which the development of colitis is prevented, we used CD4+CD45RBhigh T cells obtained from $ROR\gamma t^{gfp/+}$ mice. To this end, RAG-2−/− mice were transferred with $ROR\gamma t^{gfp/+}$ CD4+CD45RBhigh T cells (Ly5.2+) alone ($Gr.1^D$) or $ROR\gamma t^{gfp/+}$ CD4+CD45RBhigh T cells (Ly5.2+) plus CD4+CD25+ T_R cells (Ly5.1+) ($Gr.2^D$) (Figure 4*A*). As expected, $Gr.1^D$ mice developed colitis, whereas $Gr.2^D$ mice did not, as assessed by weight loss (Supplementary Figure 4*A*), clinical score (data not shown), histological appearance (data not shown), and absolute number of LP CD3+CD4+ T cells (Supplementary Figure 4*B*), in contrast to mice transferred with $ROR\gamma t^{gfp/+}$ CD4+CD45RBhigh T cells alone ($Gr.1^D$), which developed colitis. Similarly to the previous experiments (Figure 3), the percentages of Th17 and Th17/Th1 cells in $Gr.2^D$ mice were significantly higher than those in $Gr.1^D$ mice, while the ratio of Th1 cells, including Th1-like cells, in $Gr.2^D$ mice was significantly lower than in $Gr.1^D$ mice (Supplementary Figure 4*C-a* and *b*). The percentages of IL-17A+CTLA-4−, IL-17A+CTLA-4+, and IL-17A−CTLA-4+ subpopulations in noncolitic $Gr.2^D$ mice were significantly higher than those in $Gr.1^D$ mice (Supplementary Figure 4*D-a* and *b*). The percentage of GFP+ Ly5.2+CD3+CD4+ T cells in $Gr.2^D$ mice was markedly up-regulated (Figure 4*B*), and was statistically higher

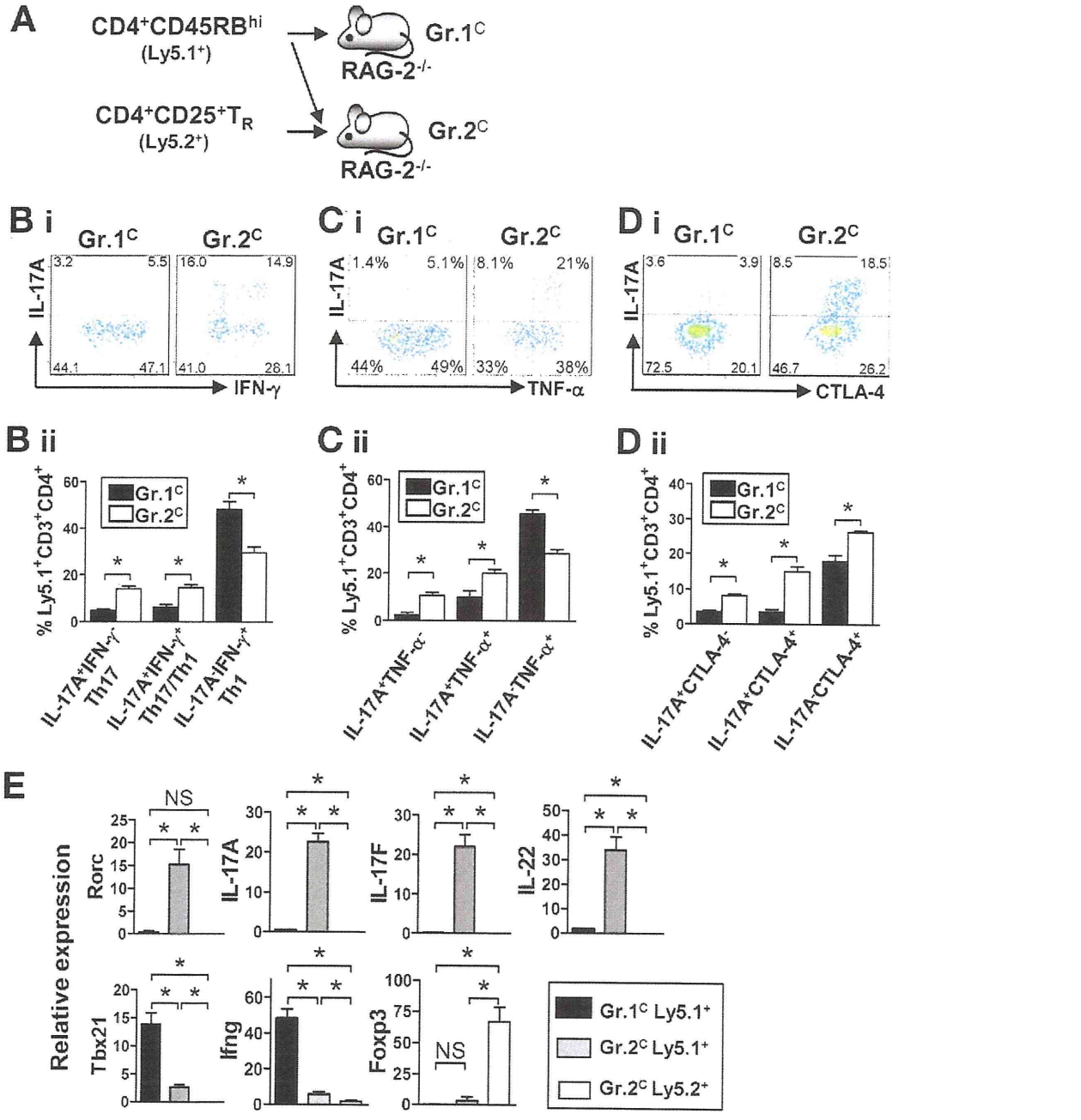
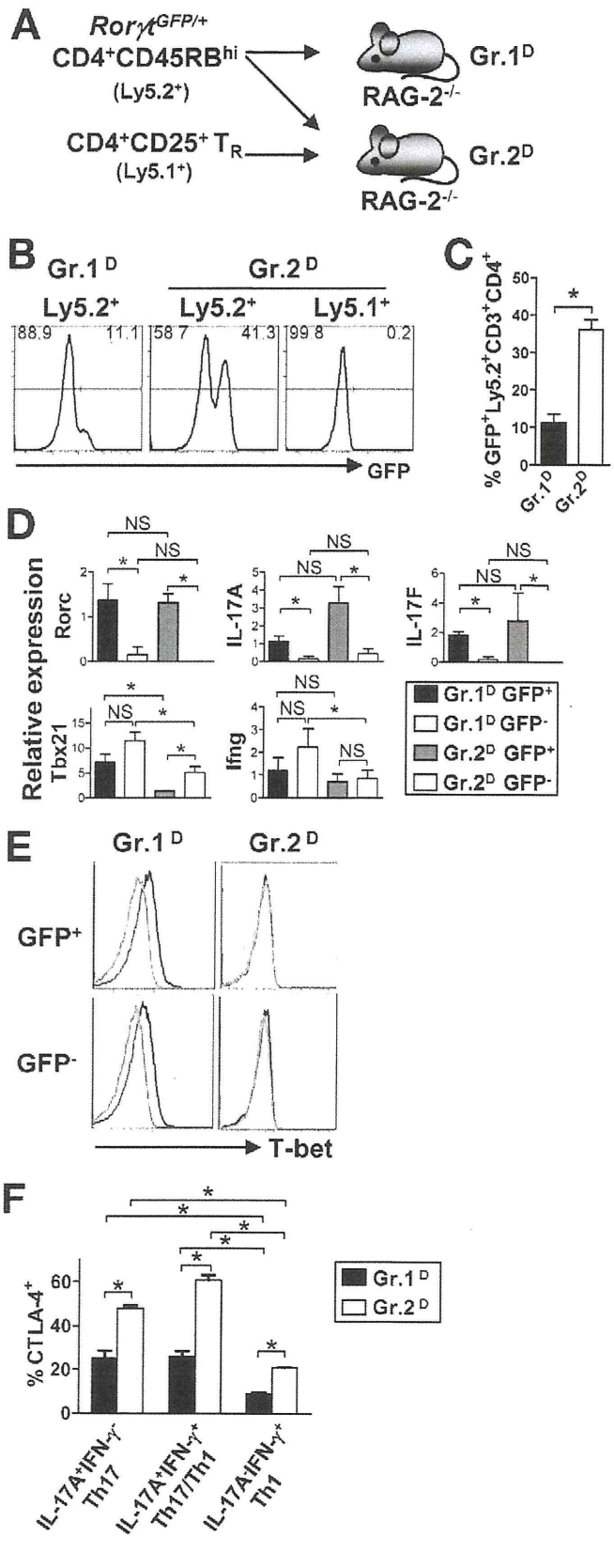


Figure 3. T_R cells not only suppress the development of colitis but also induce the accumulation of Th17 and Th17/Th1 cells in noncolitic mice. (*A*) Transfer protocol C. RAG-2$^{-/-}$ mice were transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells alone (Gr.1^C, n = 6) or Ly5.1$^+$CD4$^+$CD45RB^{high} T cells plus Ly5.2$^+$CD4$^+$CD25$^+$ T_R cells (Gr.2^C, n = 6). Mice were sacrificed 7 weeks after transfer. (*B-i*) Expression of IL-17A and IFN-γ in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of 6 mice in each group. (*B-ii*) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in colonic CD3$^+$CD4$^+$ T cells. (*C-i*) Expression of IL-17A and TNF-α in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of six mice in each group. (*C-ii*) Mean percentages of IL-17A$^+$TNF-α$^-$, IL-17A$^+$TNF-α$^+$, and IL-17A$^-$TNF-α$^+$ in colonic CD3$^+$CD4$^+$ T cells. (*D-i*) Expression of IL-17A and CTLA-4 in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of 6 mice in each group. (*D-ii*) Mean percentages of IL-17A$^+$CTLA-4$^-$, IL-17A$^+$CTLA-4$^+$, and IL-17A$^-$CTLA-4$^+$ in colonic CD3$^+$CD4$^+$ T cells. (*E*) Expression of the indicated mRNA in colonic Ly.5.1$^+$CD3$^+$CD4$^+$ T cells, normalized to Act-b expression. Statistical data (*B-ii, C-ii, D-ii, E*) show mean ± standard error of mean (n = 6/group). *$P < .05$. NS, not significant.

when compared to Gr.1^D mice (Figure 4*C*). However, the absolute number of GFP$^+$Ly5.2$^+$CD3$^+$CD4$^+$ T cells in Gr.2^D mice was comparable to that in Gr.1^D mice (Supplementary Figure 4*F*), because the absolute number of LP CD3$^+$CD4$^+$ T cells in Gr.1^D mice with colitis was significantly increased as compared to that in noncolitic Gr.2^D mice (Supplementary Figure 4*B*).

Real-time quantitative polymerase chain reaction analysis revealed that GFP$^-$ CD3$^+$CD4$^+$ T cells in colitic Gr.1^D mice dominantly express Tbx21, but not Rorc, while GFP$^+$ CD3$^+$CD4$^+$ T cells in those mice express not only Rorc, but also Tbx21 (Figure 4*D*), suggesting that RORγt$^+$T-bet$^+$ CD3$^+$CD4$^+$ T cells reside in the GFP$^+$CD3$^+$CD4$^+$ T cells of colitic mice. In noncolitic

$Gr.2^D$ mice, $GFP^+Ly5.2^+CD3^+CD4^+$ T cells dominantly expressed Rorc, but not Tbx21, while $GFP^-Ly5.2^+CD3^+CD4^+$ T cells did not express Rorc, but slightly expressed Tbx21 (Figure 4*D*). Importantly, T-bet protein was slightly, but significantly, detected in all LP $CD3^+CD4^+$ T cells in both GFP^+ and GFP^- $CD3^+CD4^+$ populations from colitic $Gr.1^D$ mice, whereas its expression was blocked in LP GFP^+ and GFP^- $CD3^+CD4^+$ populations from noncolitic $Gr.2^D$ mice (Figure 4*E*). The histogram shows 1 peak shift compared to the isotype control, suggesting that all cells are T-bet–positive, but we cannot rule out a possibility that there may be cells within these populations that do not express T-bet. Interestingly, CTLA-4 expression in Th17, Th17/Th1, and Th1 cells obtained from the LP of noncolitic $Gr.2^D$ mice was significantly higher than the corresponding cells obtained from the LP of colitic $Gr.1^D$ mice (Figure 4*F*, Supplementary Figure 4*G*). Furthermore, CTLA-4 expression in Th17 and Th17/Th1 was significantly higher than the paired Th1 cells in both colitic $Gr.1^D$ mice and noncolitic $Gr.2^D$ mice (Figure 4*F*, Supplementary Figure 4*G*). In addition, we also found that a portion of T_R cells in $Gr.2^D$ mice converted into IFN-γ–expressing T cells (Supplementary Figure 4*E*).



T_R Cells Suppress Development of Colitis Induced by Colitogenic RORγt$^+$ or RORγt$^-$ CD4$^+$ T Cells Resided in Noncolitic Mice

We next investigated whether $ROR\gamma t^{gfp/+}$ $CD4^+$ $CD45RB^{high}$ T-cell–derived RORγt$^+$ (GFP^+) or RORγt$^-$ (GFP^-) $CD4^+$ T cells residing in noncolitic RAG-$2^{-/-}$ mice in which the development of colitis is prevented by cotransfer of $CD4^+CD25^+$ T_R cells ($Ly5.1^+$) with $ROR\gamma t^{gfp/+}$ $CD4^+CD45RB^{high}$ T cells ($Ly5.2^+$) have the potential to induce colitis when they are separated from the T_R population and retransferred to new RAG-$2^{-/-}$ mice as depicted in Figure 5*A*. Although $Gr.3^E$ mice, which were transferred with non-Th17 RORγt$^-$ (GFP^-) cells, showed marked weight loss, $Gr.1^E$ mice, which were transferred with a mixture of Th17 cells and Th17/Th1 RORγt$^+$ (GFP^+) cells, did not, indicating that the weight loss in Gr. 3^E mice was significantly lower than in other groups (Figure 5*B*). This suggests that wasting disease is mediated by non-Th17 cells (possibly Th1 cells). Nevertheless, the colons of both $Gr.1^E$ and $Gr.3^E$ mice, but not $Gr.2^E$ and $Gr.4^E$ mice, showed similarly thickened walls (data not shown). Total clinical scores of $Gr.1^E$ and $Gr.3^E$ mice were comparably high and

Figure 4. T_R cells suppress the development of colitis with the increase of RORγt$^+$ cells. (*A*) Transfer protocol D. RAG-$2^{-/-}$ mice were transferred with $Ly5.2^+$ $ROR\gamma t^{gfp/+}$ $CD4^+CD45RB^{high}$ T cells alone ($Gr.1^D$, n = 6) or $Ly5.2^+$ $ROR\gamma t^{gfp/+}$ $CD4^+CD45RB^{high}$ T cells plus $Ly5.1^+CD4^+CD25^+$ T_R cells ($Gr.2^D$, n = 6). Mice were sacrificed 6 weeks after transfer. (*B*) Expression of GFP in colonic $Ly5.2^+$ or $Ly5.1^+$ $CD3^+CD4^+$ T cells. Data are representative of 6 mice in each group. (*C*) Percentage of GFP^+ cells in colonic $Ly5.2^+$ $CD3^+CD4^+$ T cells. (*D*) Expression of the indicated mRNA in GFP^+ or GFP^- $CD3^+CD4^+$ cells obtained from the colonic $Ly.5.2^+$ $CD3^+CD4^+$ T cells of $Gr.1^D$ and $Gr.2^D$ mice, normalized to Act-b expression. (*E*) Expression of T-bet in GFP^+ or GFP^- colonic $Ly5.2^+CD3^+CD4^+$ T cells of each group. Data are representative of 6 mice in each group. (*F*) Mean percentages of CTLA-4 in colonic IL-17A$^+$IFN-γ$^-$ Th17, IL-17A$^+$IFN-γ$^+$ Th17/Th1, and IL-17A$^-$IFN-γ$^+$ Th1 cells. Statistical data (*C, D, F*) show mean ± standard error of mean (n = 6/group). *$P < .05$. NS, not significant.

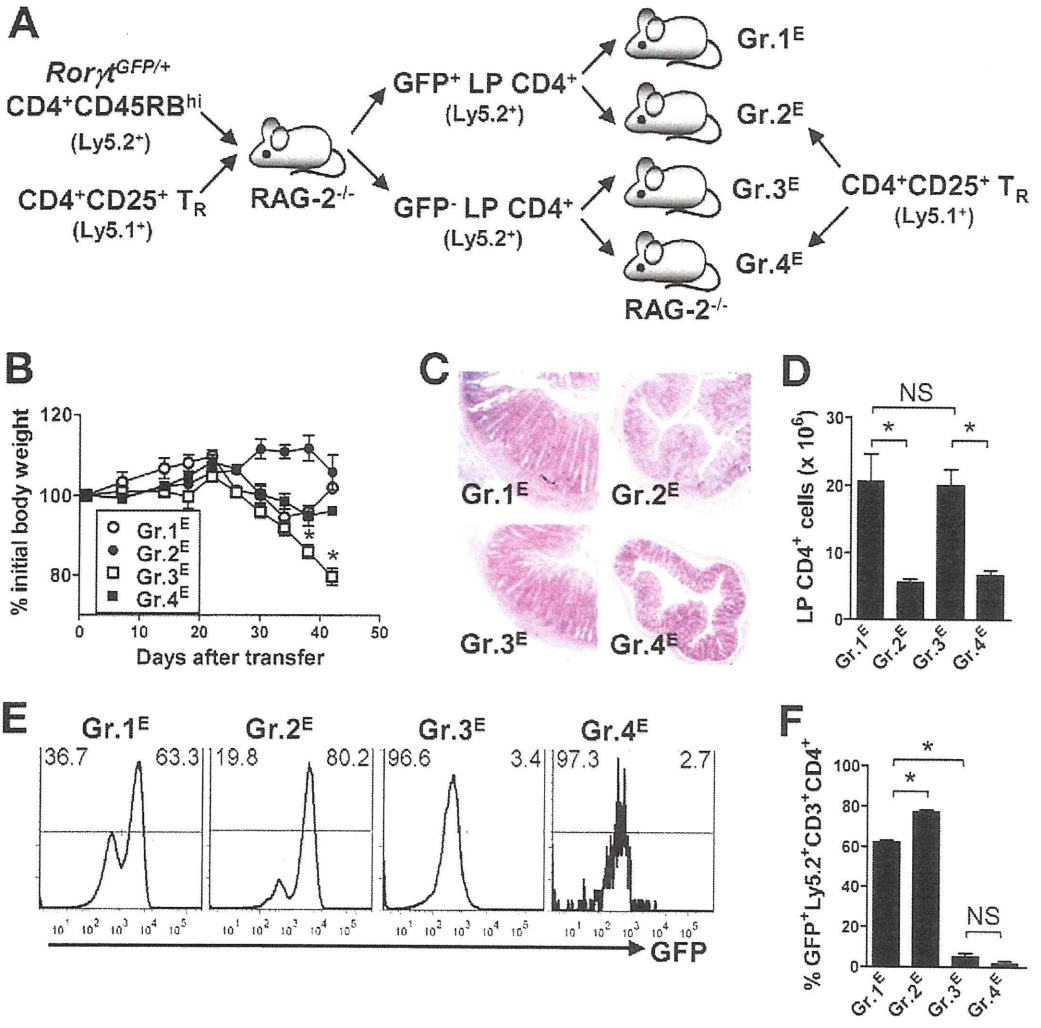


Figure 5. T_R cells suppress the development of colitis induced by RORγt$^+$ or RORγt$^-$ CD4$^+$ T cells resided in noncolitic mice. (*A*) Transfer protocol E. RAG-2$^{-/-}$ mice were transferred with GFP$^+$ or GFP$^-$ Ly5.2$^+$CD4$^+$ T cells obtained from noncolitic mice previously transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells and Ly5.1$^+$CD4$^+$CD25$^+$ T_R cells with or without cotransfer of freshly isolated Ly5.1$^+$CD4$^+$CD25$^+$ T cells from normal mice (Gr.1^E-Gr.4^E, each n = 6). Mice were sacrificed 6 weeks after transfer. (*B*) Change in body weight. *$P < .05$. (*C*) Histopathology of distal colon at 6 weeks after transfer. Original magnification, ×40. (*D*) Absolute cell number of recovered colonic LP CD3$^+$CD4$^+$ T cells at 6 weeks after transfer. (*E*) Expression of GFP in colonic Ly5.2$^+$CD3$^+$CD4$^+$ T cells of each group. Data are representative of 6 mice in each group. (*F*) Mean percentages of GFP expression in colonic Ly5.2$^+$CD3$^+$CD4$^+$ T cells of each group. Data (*D, F*) show mean ± standard error of mean (n = 6/group). *$P < .05$. NS, not significant.

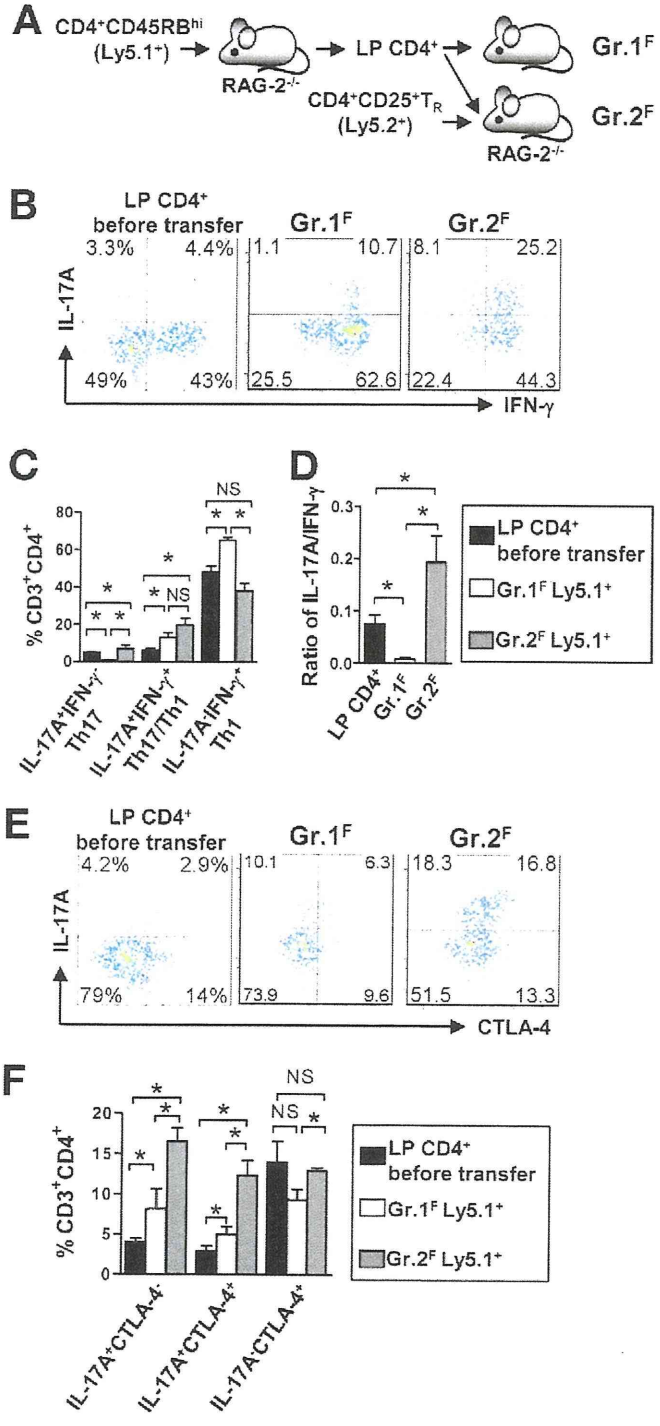
higher than the paired Gr. 2^E and Gr.4^E mice, respectively (Supplementary Figure 5*A*). Consistent with this, Gr.1^E and Gr.3^E mice, but not Gr.2^E and Gr.4^E mice, microscopically developed severe colitis with a marked increase of infiltrating mononuclear cells in the colon, in sharp contrast to Gr.2^E and Gr.4^E mice with no colitis (Figure 5*C*). The histological scores of colitic Gr.1^E and Gr.3^E mice were comparable, but were significantly higher than the paired controls, Gr.2^E and Gr.4^E, respectively (Supplementary Figure 5*B*). Although the absolute cell number of LP CD3$^+$CD4$^+$ T cells in Gr.1^E and Gr.3^E was equivalent, significantly higher numbers of CD3$^+$CD4$^+$ T cells were recovered from the LP of Gr.1^E and Gr.3^E mice than from the paired Gr.2^E and Gr.4^E mice, respectively (Figure 5*D*).

Given the evidence that both LP GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells in noncolitic mice previously transferred with CD4$^+$CD45RB^{high} T cells and T_R cells are colitogenic if they are separated from T_R cells and transferred to new RAG-2$^{-/-}$ mice, we next examined the plasticity of those cells after the second transfer. Although LP Ly5.2$^+$GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.3^E and Gr.4^E mice did not gain GFP expression after retransfer, regardless of whether colitis developed, approximately one third of LP CD3$^+$CD4$^+$ T cells derived from Ly5.2$^+$GFP$^+$ T cells in Gr.1^E with colitis lost the expression of GFP, and this loss in LP Ly5.2$^+$CD3$^+$CD4$^+$ T cells of Gr.2^E mice was blocked by cotransfer of T_R cells (Figure 5*E* and *F*), suggesting that T_R cells suppress the differentiation from RORγt$^+$ Th17 and Th17/Th1 cells to RORγt$^-$ Th1 cells during colitis development.

T_R Cells Modulate the Balance Between Th17 and Th1 Cells

We finally assessed if T_R cells remodulate the balance of Th17, Th17/Th1, and Th1 cells after colitis is established. To this end, we prepared colitic RAG-2$^{-/-}$ mice previously transferred with Ly5.1$^+$CD4$^+$CD45RB^{high}

T cells. Eight weeks after transfer, RAG-$2^{-/-}$ mice were transferred with LP Ly.5.1$^+$CD3$^+$CD4$^+$ T cells obtained from the established colitic mice with (Gr.2^F) or without (Gr.1^F) Ly5.2-derived T_R cells (Figure 6*A*). First, we confirmed that Gr.1^F mice developed colitis, while Gr.2^F mice cotransferred with T_R cells developed significantly milder colitis as compared to Gr.1^F mice, as assessed by weight loss (Supplementary Figure 6*A*), clinical score (Supplementary Figure 6*B*), colon appearance (data not shown), histology (Supplementary Figure 6*C*), and the absolute number of LP CD3$^+$CD4$^+$ T cells recovered (Supplementary Figure 6*D*). Furthermore, the percentage of Th17 cells in the LP CD3$^+$CD4$^+$ T cells of colitic Gr.1^F mice was significantly reduced compared to the original mice with colitis, while conversely, the percentages of Th17/Th1 and Th1 cells in Gr.1^F mice were significantly higher when compared to those of the original mice with colitis, suggesting a sequential developmental pathway from Th17 to Th17/Th1 and Th1 cells after retransfer. In contrast, the percentage of Th1 cells in the LP CD3$^+$CD4$^+$ T cells of Gr.2^F mice was significantly lower than in Gr.1^F mice, while the percentages of Th17 cells in the LP CD3$^+$CD4$^+$ T cells of Gr.2^F mice were significantly higher than in Gr.1^F mice (Figure 6*B* and *C*). Therefore, the ratio of Th17 to Th1 cells in Gr.2^F mice was markedly higher than that in Gr.1^F mice (Figure 6*D*), indicating that cotransfer of T_R cells skews the balance of Th17 and Th1 cells from Th1 to Th17 cells. The percentage of Th17/Th1 cells in Gr.2^F mice tended to be higher than that in Gr.1^F mice, but the difference was not significant (Figure 6*C*). The CTLA-4 expression of both IL-17A$^+$ and IL-17A$^-$ CD4$^+$ T cells in Gr.2^F mice was significantly increased as compared to the paired percentages in Gr.1^F mice (Figure 6*E* and *F*). These results suggest that T_R cells suppress the maintenance of Th1 cells, or induce the re-differentiation from Th1 to Th17 cells.



Discussion

In the present study, we demonstrated that colitogenic RORγt$^+$ CD4$^+$ T cells reside in inflamed mucosa dominantly as Th1-like cells that exclusively express IFN-γ, rather than Th17/Th1 cells expressing both IFN-γ and IL-17A; these Th1-like cells developmentally lose RORγt expression and terminally differentiate into RORγt$^-$T-bet$^+$ Th1 cells that exclusively express IFN-γ, which we designate "alternative Th1 cells"; and as a highlight finding, T_R cells suppress development of colitis by blocking the developmental pathway from Th17 to alternative Th1 cells via Th17/Th1 and Th1-like cells.

Figure 6. T_R cells modulate the balance between Th17 and Th1 cells. (*A*) Transfer protocol F. RAG-$2^{-/-}$ mice were transferred with colitogenic CD4$^+$ T cells obtained from colitic mice previously transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells with (Gr.2^F, n = 6) or without (Gr.1^F, n = 6) Ly5.2$^+$CD4$^+$CD25$^+$ T_R cells. Mice were sacrificed 6 weeks after transfer. (*B*) Expression of IL-17A and IFN-γ in the originally transferred LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells and Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the colon. Data are representative of 6 mice in each group. (*C*) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in the originally transferred LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells and Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the colon of Gr.1^F or Gr.2^F mice. (*D*) Ratio of Th17 to Th1 cells in LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells. (*E*) Expression of IL-17A and CTLA-4 in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of six mice in each group. (*F*) Mean percentages of IL-17A$^+$CTLA-4$^-$, IL-17A$^+$CTLA-4$^+$, and IL-17A$^-$CTLA-4$^+$ in Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the originally transferred LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells and Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the colon of Gr.1^F or Gr.2^F mice. Statistical data (*C, D, F*) show mean ± standard error of mean (n = 6/group). *$P < .05$.

BASIC AND

Recent reports demonstrated that Th17 cells have an ability to divert to IFN-γ–producing cells in vitro, or via a combined system using sequential in vitro and in vivo experiments and vice versa.[10,19,20] The current study proposes a linear developmental pathway of alternative Th1 cells in the development of colitis from naïve to Th17, Th17/Th1, and Th1-like cells and then to alternative Th1 cells, in addition to a developmental pathway of classical Th1 cells (Supplementary Figure 7). However, it still remains to be determined whether the sequential path from naïve to Th17 to Th17/Th1 to Th1-like to alternative Th1 cells occurs during the development of colitis, as we did not analyze the data using an IL-17A/F, or IFN-γ/T-bet reporter in this project. Further studies will be needed to address this issue.

Results of our current project show some similarities to the findings reported by Weaver's group,[10] but the experimental protocols and interpretations of the 2 studies are quite different. Weaver's group used in vitro–induced IL-17F$^+$ Th17 cells sorted from naïve IL-17F reporter CD4$^+$ T cells that had been stimulated with TGF-β and IL-6 as donor cells, and then transferred those cells into RAG-1$^{-/-}$ mice. In this setting, mice not only developed colitis, but also retained IL-17A$^-$IFN-γ$^+$ CD4$^+$ T cells in the inflamed mucosa. They concluded that committed Th17 cells give rise to progeny that lost IL-17A expression and up-regulated IFN-γ expression at the late stage of colitis development. They called such cells "Th1-like" cells. However, they did not characterize the expression of RORγt and T-bet in IL-17A$^-$IFN-γ$^+$ CD4$^+$ T cells in the inflamed mucosa of colitic RAG-1$^{-/-}$ mice transferred with in vitro–induced Th17 cells, but did demonstrate that in vitro–induced Th17 cells with RORγt, but not T-bet, expression convert to "Th1-like" cells with T-bet, but not RORγt, expression in all in vitro stimulation systems. In our project, however, we performed a series of studies using a consecutive in vivo adoptive transfer system, including the induction stage of Th17 cells and the late stage. Therefore, unlike Weaver's group, we characterized in vivo pathologically occurring RORγt$^+$ Th17 or Th17/Th1 cells in colitic mice, and used those cells rather than the in vitro–stimulated Th17 cells as donor cells. In this setting, the majority of RORγt$^+$ CD3$^+$CD4$^+$ T cells in the colitic mice were composed of IL-17A$^-$IFN-γ$^+$ CD4$^+$ T cells that express both Rorc and Tbx21 and a few IL-17A$^+$IFN-γ$^+$ Th17/Th1 and IL-17A$^+$IFN-γ$^-$ Th17 cells. Importantly, additional adoptive transfer of RORγt$^+$ CD3$^+$CD4$^+$ T cells into RAG-2$^{-/-}$ mice demonstrated that approximately half of the transferred RORγt$^+$ cells lose their RORγt expression and gained T-bet, while the other half retain it up to 6 weeks after the retransfer, suggesting that RORγt$^+$ Th17, Th17/Th1, and Th1-like cells in colitic mice act as colitogenic "memory stem-like" cells for the generation of terminally differentiated Th1 cells.

In such a linear in vivo developmental pathway of Th17 and Th17/Th1 to Th1-like and alternative Th1 cells, we strikingly demonstrated that T_R cells suppress the transition of Th17/Th1 into Th1-like cells (Figure 3 and 4, Supplementary Figure 6), as paradoxically the ratio of Th17 and Th17/Th1 cells is significantly higher in noncolitic RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T and T_R cells than RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells alone. Indeed, although almost all previous reports conclude that T_R cells suppress production of both IFN-γ and IL-17A in this model[21,22] and other animal models of chronic inflammation,[23] the conclusion is based on the assessment of the production of IL-17A by anti-CD3 monoclonal antibody stimulation or expression of IL-17A mRNA in the intestinal mucosa. The current intracellular staining method that precisely discriminates effector and T_R cells using the Ly5.1/Ly5.2 congenic system may make it possible to draw a surprising conclusion. The finding that cotransfer of T_R cells resulted in an increased ratio of Th17 and Th17/Th1, but a decreased ratio of Th1, indicates that T_R cells block the transition of Th17/Th1 cells into Th1-like cells.

Furthermore, the role of T_R cell–mediated accumulation of Th17 and Th17/Th1 cells should be discussed in the context of a recently published article by Ghoreschi et al.[24] They emphasize that the presence and absence of TGF-β determines the fate of the subsequent development of regulatory and pathological Th17 cells, respectively, in a model of experimental autoimmune encephalomyelitis. According to this article and the previous finding that T_R cells produce TGF-β,[25] our data suggest that T_R cells instruct development of regulatory Th17 cells in noncolitic mice cotransferred with T_R cells. Consistently, regardless of the increased Th17 and Th17/Th1 cells or decreased Th1 cells, expression of CTLA-4 in those cells of noncolitic mice cotransferred with T_R cells was significantly up-regulated, indicating that T_R cells suppress colitis by not only suppressing development of Th1 cells, but also inducing the inhibitory molecule, CTLA-4, in effector T cells. Given the finding that T_R cells require their CTLA-4 expression to protect from colitis,[26] CTLA-4 not only in T_R but also in effector cells may play an important role in inducing inhibitory signaling through CD80/CD86 in noncolitic conditions. However, this scenario seems to be unlikely because we showed that the ratio of IL-17A$^+$TNF-α$^+$ cells in noncolitic mice cotransferred with T_R cells was significantly higher than that of paired cells in colitic mice; and Th17 and Th17/Th1 cells in noncolitic mice cotransferred with T_R cells are precolitogenic (Figure 5). Therefore, the mechanism of the developmental pathways of Th17 cells in the colitis model and encephalomyelitis model may be distinct.

Collectively, the current study solves the riddle of whether Th1 or Th17 cells are essential for development of colitis. Our answer is both, because Th17 cells may be precursor cells for alternative Th1 cells. Therefore, T-bet, which is involved in the functionality of both classical and alternative Th1 cells, may be a feasible target for the treatment of IBD.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.05.052.

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Reprint requests

Address requests for reprints to: Takanori Kanai, MD, PhD, and Toshifumi Hibi, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 180-8582, Japan. e-mail: takagast@sc.itc.keio.ac.jp **and** thibi@sc.itc.keio.ac.jp**; fax: +81-3-3341-3631.**

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S.T. and Y.O. contributed equally to this work.

Conflicts of interest

The authors disclose no conflicts.

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