as IL-17A, IL-17F, and IL-22 participate in the pathogenesis of RA (6,7). IL-17A levels have been shown to be elevated in synovium and synovial fluid from RA patients (8,9). In addition, it has been shown that the number of CD4+ T cells with a Th17 cell phenotype (CCR6+IL-17A+TNFα+ memory T cells) is increased in untreated RA patients as compared with healthy controls (10). Intriguingly, when these Th17 cell–like CD4+ T cells are cocultured with synovial fibroblasts, they produce not only IL-17A, but also proinflammatory cytokines and tissue-destructive enzymes (10). Furthermore, the induction of collagen-induced arthritis, a murine model of RA, is attenuated in mice lacking IL-17A (11) or IL-23 (12), the latter of which promotes the differentiation of pathogenic Th17 cells in conjunction with IL-6 and transforming growth factor β (TGFβ) (13). These findings indicate that Th17 cells play a pivotal role in the pathogenesis of RA.

During the differentiation of Th17 cells, IL-6/STAT-3 signaling along with TGFβ induces the expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt), which functions as a lineage-specifying transcription factor of Th17 cells (13). Consistently, T cells lacking STAT-3 exhibit reduced expression of RORγt and impaired Th17 cell differentiation (14). These findings are consistent with the clinical efficacy of tocilizumab (TCZ), an anti–IL-6 receptor monoclonal antibody (mAb) that blocks IL-6/STAT-3 signaling, in RA (15). However, detailed mechanisms underlying the efficacy of IL-6 blockade for RA are not fully understood. In the present study, we examined gene expression profiles of CD4+ T cells before and after TCZ therapy in RA patients who showed good clinical responses to the therapy to clarify the mechanisms underlying the efficacy of IL-6 blockade in RA.

PATIENTS AND METHODS

Patients. Patients who fulfilled the American College of Rheumatology 1987 revised classification criteria for RA (16) were recruited to the study when they and their physicians intended to start TCZ therapy as routine care for uncontrolled arthritis between June 2009 and October 2011. Patients attending the Department of Allergy and Clinical Immunology, Chiba University Hospital and the Research Center for Allergy and Clinical Immunology, Asahi General Hospital were studied. For control groups, treatment-naive RA patients, RA patients treated with TNF inhibitors (TNFi) or abatacept, and age-matched healthy controls were recruited. The patients' disease status was assessed at baseline and at week 24 of therapy using the Clinical Disease Activity Index (CDAI) score (17). Good clinical response to therapy was defined as an improvement of >50% from the baseline CDAI score. The entire study was approved by the Ethics Committees of Chiba University, Asahi General Hospital, and Kazusa DNA Research Institute and was performed in accordance with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all study subjects.

Isolation of human CD4+ T cells. Mononuclear cells were isolated from peripheral blood by Ficoll-Paque density-gradient centrifugation. CD4+ T cells were purified from mononuclear cells using a CD4+ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of CD4+ T cells was routinely >98% by fluorescence-activated cell sorting (FACS) analysis.

DNA microarray analysis. Total cellular RNA was extracted from human CD4+ T cells with Isogen solution (Nippon Gene). DNA microarray analysis was performed using a Quick Amp Labeling kit (Agilent) and a Whole Human Genome DNA Microarray 4×44K (Agilent) according to the manufacturer's protocols. Microarray data were analyzed using GeneSpring GX11.5.1 software (Agilent).

Mice and reagents. C57BL/6 mice were purchased from Charles River Laboratories. RORγt-deficient mice (13) were kind gifts from Dr. Y. Iwakura (Tokyo University of Science, Tokyo, Japan). All mice were housed in microisolator cages under specific pathogen–free conditions. Animal procedures used in this study were approved by the Chiba University Animal Care and Use Committee.

Antibodies to murine CD3ε (145-2C11), CD28 (37.51), IL-4 (11B11), and interferon-γ (IFNγ) (XMG1.2) were purchased from BD Biosciences. Murine IL-4 and IL-6 were purchased from PeproTech. Human TGFβ was purchased from R&D Systems. STAT-3 inhibitor VI (S3I-201) was purchased from Santa Cruz Biotechnology.

Plasmids. Complementary DNAs (cDNAs) for murine AT-rich–interactive domain–containing protein 5A (ARID-5A) and RORγt were subcloned into pMX-IRES-GFP vector and MSCV-IRES-Thy1.1 vector, respectively. Truncated mutants of ARID-5A were generated using a KOD-Plus Mutagenesis kit (Toyobo) as described previously (18). Murine IL-17A promoter (19) was subcloned into the pGL3 vector to generate −153 mIL17p-Luc or −94 mIL17p-Luc. All sequences were verified by DNA sequencing.

Cell culture. Murine naive CD4+ T cells (CD62L^{high} CD25−CD4+ T cells) were isolated from spleen and lymph nodes using a CD4+ T Cell Isolation Kit II according to the manufacturer's instructions. The purity of isolated cells was routinely >98% by FACS analysis. Naive CD4+ T cells (5×10^5/ml) were stimulated with plate-bound anti-CD3ε mAb (1 μg/ml) plus anti-CD28 mAb (2 μg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and antibiotics at 37°C. Where indicated, IL-6 (100 ng/ml), TGFβ (1 ng/ml), anti–IL-4 mAb (5 μg/ml), and anti-IFNγ mAb (5 μg/ml) were added to induce Th17 cells (Th17 cell–polarizing conditions). Anti–IL-4 mAb (5 μg/ml) and anti-IFNγ mAb (5 μg/ml) were added to induce Th0 cells (Th0 cell conditions). IL-6 (100 ng/ml), IL-23 (100 ng/ml), anti-TGFβ mAb (5 μg/ml), anti–IL-4 mAb (5 μg/ml), and anti-IFNγ mAb (5 μg/ml) were added to induce Th22 cells (Th22 cell–polarizing conditions) (20). TGFβ (1 ng/ml) was added to induce Treg cells (Treg cell–polarizing conditions).

Induction of human Th17 cells. Memory CD4+ T cells were isolated from peripheral blood mononuclear cells

of healthy controls or untreated RA patients using a Human Memory CD4+ T Cell Isolation kit (Miltenyi Biotec). They were then stimulated for 14 days with plate-bound anti-CD3 antibody (1 μg/ml; eBioscience) plus anti-CD28 antibody (1 μg/ml; eBioscience) under Th0 cell conditions (IL-2 [10 ng/ml], anti–IL-4 antibody [5 μg/ml], and anti-IFNγ antibody [5 μg/ml]) or Th17 cell–polarizing conditions (IL-6 [10 ng/ml], IL-23 [10 ng/ml], IL-1β [10 ng/ml], IL-2 [10 ng/ml], anti–IL-4 antibody [5 μg/ml], and anti-IFNγ antibody [5 μg/ml]).

Real-time polymerase chain reaction (PCR) analysis. Total cellular RNA was extracted from human and murine CD4+ T cells with Isogen solution, and reverse transcription was carried out using an iScript cDNA Synthesis kit (Bio-Rad). The expression of ARID-5A was measured by real-time quantitative PCR analysis using a standard protocol on an ABI Prism 7300 instrument (Applied Biosystems). The levels of ARID-5A were normalized to the levels of GAPDH.

Retrovirus-mediated gene induction. Retrovirus-mediated gene induction for murine naive CD4+ T cells was performed as described previously (21).

Intracellular staining. Cultured cells were harvested and restimulated with phorbol myristate acetate (20 ng/ml) plus ionomycin (1 μg/ml) at 37°C for 5 hours in the presence of monensin (2 μ*M*; Sigma). Intracellular staining for murine IL-17A, IL-17F, IL-22, and FoxP3 was performed as described previously (22).

Western blotting. Whole cell lysates were prepared, and immunoblotting was performed as described previously (23). Anti-human ARID-5A polyclonal antibody was purchased from Abcam.

Immunoprecipitation assay. Using Lipofectamine (Invitrogen), we transfected 293T cells with either pcDNA3-FLAG-ARID5A (wild-type [WT]) or the truncated mutants of ARID-5A and/or MSCV-myc-RORγt-IRES-Thy1.1. Cells were lysed with lysis buffer, and the lysates were incubated with anti-FLAG M2-Agarose Affinity Gel (Sigma) for 2 hours at 4°C. After washing, samples were subjected to Western blotting with horseradish peroxidase (HRP)–conjugated anti-Myc antibody (9E10; Santa Cruz Biotechnology) or HRP-conjugated anti-FLAG antibody (M2; Sigma).

Luciferase assay. EL4 cells (5×10^5) were transfected with the indicated plasmids using a Neon Transfection System (Life Technologies) according to the manufacturer's instructions. Twenty-four hours later, a luciferase reporter assay was performed with a dual luciferase assay system (Promega) according to the manufacturer's instructions. All values were obtained from experiments carried out in triplicate and repeated at least 3 times.

Statistical analysis. Data are reported as the mean ± SD. Statistical analysis was performed using analysis of variance or unpaired *t*-test as appropriate. *P* values less than 0.05 were considered significant.

RESULTS

Reduced expression of ARID-5A in CD4+ T cells from RA patients with good clinical responses to TCZ therapy. Ten RA patients who received TCZ therapy for uncontrolled arthritis were enrolled in this study. Eight of them showed good clinical responses to TCZ therapy as evaluated by an improvement of >50% from the baseline CDAI score (further information is available at http://www.m.chiba-u.jp/class/allergy/). To clarify the mechanisms underlying the efficacy of IL-6 blockade for RA, we examined gene expression profiles of CD4+ T cells by DNA microarray analysis at baseline and at 12 weeks of TCZ therapy in the RA patients who showed good clinical responses to the therapy. Analysis of microarray data using a weighted average difference method (24) identified several signaling molecules and transcription factors whose expression was significantly reduced in CD4+ T cells by TCZ therapy (Table 1). The identification of several known IL-6/STAT-3– or Th17 cell–related genes, such as SOCS3 (25,26), BCL3 (27), and BATF (28), supported the reliability of this screening. We identified ARID5A (also known as MRF1) as one of the new genes down-regulated by IL-6 blockade in the form of TCZ therapy (Table 1).

Table 1. Weighted average difference ranking of differentially expressed genes in CD4+ T cells from rheumatoid arthritis patients who responded to tocilizumab therapy

Weighted average difference rank	Gene name	Regulation from 0 weeks to 12 weeks	Absolute fold change	Probe name
1	SOCS3	Down	7.5	A_23_P207058
2	BCL3	Down	2.7	A_23_P4662
3	BATF	Down	2.5	A_23_P128974
4	MYC	Down	2.2	A_23_P215956
5	PIM1	Down	1.9	A_23_P345118
6	ARID5A	Down	2.1	A_23_P143016
7	SOCS1	Down	2.3	A_23_P420196
8	PIM3	Down	1.7	A_23_P61398

First, we compared the expression of ARID5A in CD4+ T cells between untreated RA patients (n = 17) and healthy controls (n = 10). The ARID5A signals were significantly higher in untreated RA patients than in healthy controls ($P < 0.01$) (Figure 1A). Consistent with the findings of analysis by the weighted average difference method, the signals of ARID5A in CD4+ T cells were significantly decreased by TCZ therapy in RA patients who showed good clinical responses to the therapy (Figure 1B), whereas they were not decreased in patients who did not (data not shown). Importantly, the ARID5A signals in CD4+ T cells were not significantly decreased in RA patients who were treated with TNFi (n = 13) or abatacept (n = 12) (Figure 1B), although these patients showed good clinical responses to the therapy, as evaluated by the CDAI (further information is available at http://www.m.chiba-u.jp/class/allergy/). These results suggest that the reduction of ARID5A

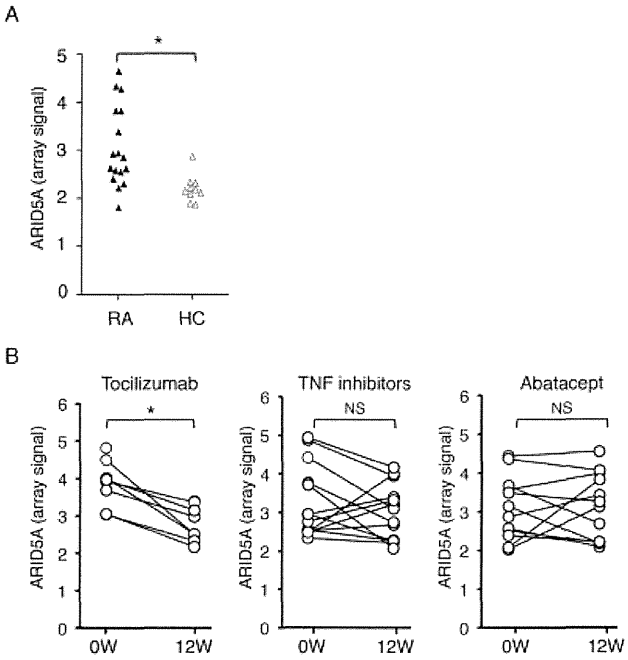

Figure 1. Expression of AT-rich–interactive domain–containing protein 5A (ARID-5A) in CD4+ T cells from rheumatoid arthritis (RA) patients is decreased by tocilizumab (TCZ) therapy. **A,** CD4+ T cells from untreated RA patients (n = 17) and those from healthy controls (HC) (n = 10) were subjected to DNA microarray analysis. Shown are array signals of ARID5A. **B,** Just before and 12 weeks after treatment, CD4+ T cells were isolated from RA patients who showed good clinical responses to treatment with TCZ (n = 8), tumor necrosis factor (TNF) inhibitors (n = 13), or abatacept (n = 12). Samples were subjected to DNA microarray analysis, and array signals of ARID5A before and after treatment were compared in each treatment group. Symbols represent samples from individual subjects. * = $P < 0.01$. NS = not significant.

expression in CD4+ T cells is specifically associated with the efficacy of TCZ therapy but not with reduced RA disease activity itself.

In addition to ARID5A, we found that the signals of some IL-6/STAT-3– or Th17 cell–related genes, such as SOCS3, BATF, NFKBIZ, and BCL3, were decreased in CD4+ T cells from RA patients who were treated with TCZ but not in those from RA patients who were treated with TNFi or abatacept (further information is available at http://www.m.chiba-u.jp/class/allergy/). In contrast, the signals of some IL-6/STAT-3– or Th17 cell–related genes including AHR and RORA were not significantly decreased in RA patients who were treated with TCZ, TNFi, or abatacept (further information is available at http://www.m.chiba-u.jp/class/allergy/). On the other hand, the signals of other IL-6/STAT-3– or Th17 cell–related genes, including IL-17A, IL-17F, IL-21, IL-23R, CCR6, and RORC, were too low to be compared in this experimental setting (data not shown).

IL-6/STAT-3 signaling induces the expression of ARID-5A in Th17 cells. To determine the roles of ARID-5A in T helper cell differentiation, we examined the expression of ARID-5A in human CD4+ T cells stimulated with anti-CD3 mAb plus anti-CD28 mAb (anti-CD3/anti-CD28) under various polarizing conditions. The expression of ARID-5A was strongly induced in CD4+ T cells from healthy controls under Th17 cell–polarizing conditions (IL-2, IL-6, IL-23, IL-1β, anti–IL-4, and anti-IFNγ) as compared with Th0 cell conditions (Figure 2A), whereas the expression of ARID-5A was not significantly induced under Th1 cell– or Th2 cell–polarizing conditions (data not shown). Consistent with the enhanced expression of ARID-5A in RA patients (Figure 1A), the expression levels of ARID-5A in CD4+ T cells under Th0 cell conditions were significantly higher in RA patients than in healthy controls (Figure 2A). However, the induction of ARID-5A expression in CD4+ T cells was not significantly different between Th0 cell conditions and Th17 cell–polarizing conditions in RA patients (Figure 2A).

We thereafter analyzed the roles of ARID-5A in T helper cell differentiation by using murine CD4+ T cells. ARID-5A was also highly expressed in murine CD4+ T cells under Th17 cell–polarizing conditions (IL-6, TGFβ, anti–IL-4, and anti-IFNγ) as compared with Th0 cell conditions (Figure 2B). The expression of ARID-5A in Th17 cells was confirmed at the protein level by immunoblotting (Figure 2C). Because ARID-5A was induced under Th17 cell–polarizing conditions, we next examined the effect of IL-6 and TGFβ (both of which are included in murine Th17 cell–polarizing conditions) on the expression of ARID-5A in CD4+ T cells. As shown in Figure 2D, IL-6, but not TGFβ, significantly induced the expression of ARID-5A in CD4+ T cells ($P < 0.05$) (n = 3 experiments). These results suggest that ARID-5A is induced in CD4+ T cells by IL-6 under Th17 cell–polarizing conditions.

To determine the signaling pathways that induce ARID-5A expression under Th17 cell–polarizing conditions, we examined the effect of an inhibitor of STAT-3 (STAT-3 inhibitor VI) on ARID-5A expression in CD4+ T cells. As shown in Figure 2E, STAT-3 inhibitor VI partially but significantly suppressed ARID-5A expression in CD4+ T cells under Th17 cell–polarizing conditions ($P < 0.05$) (n = 3 experiments). We also examined the role of RORγt, a lineage-specifying transcription factor of Th17 cells (13,29), in ARID-5A expression in CD4+ T cells by using RORγt-deficient mice. Intriguingly, ARID-5A was similarly

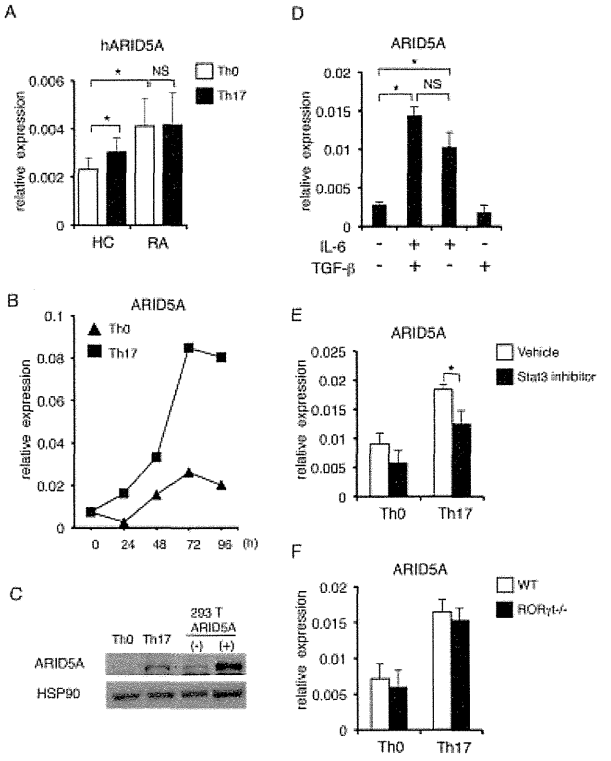

Figure 2. Interleukin-6 (IL-6)/STAT-3 signaling induces ARID-5A expression in human and murine Th17 cells. **A,** Memory CD4+ T cells from healthy controls (n = 6) or untreated RA patients (n = 6) were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions or Th17 cell–polarizing conditions, and expression of ARID-5A was measured by quantitative polymerase chain reaction (qPCR). **B,** Murine naive CD4+ T cells were stimulated under Th0 cell conditions or Th17 cell–polarizing conditions for the indicated time periods, and expression of ARID-5A was measured by qPCR. Data are representative of 3 independent experiments. **C,** Naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions or Th17 cell–polarizing conditions and were subjected to immunoblotting with anti–ARID-5A antibody. Lysates of 293T cells that were transfected with pcDNA3-ARID5A (ARID-5A+) or empty pcDNA3 (ARID-5A−) were used as controls. **D,** Naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 in the presence or absence of IL-6 and/or transforming growth factor β (TGFβ). **E,** Naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions or Th17 cell–polarizing conditions in the presence or absence of STAT-3 inhibitor VI. **F,** Naive CD4+ T cells from retinoic acid receptor–related orphan nuclear receptor γt (RORγt)–deficient mice or their wild-type (WT) littermates were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions or Th17 cell–polarizing conditions. ARID-5A expression was measured by qPCR. In **A,** values are the mean ± SD of 6 RA patients and 6 healthy controls; in **B,** values are representative (we repeated this experiment 3 times); in **D–F,** values are the mean ± SD of 3 experiments. * = $P < 0.05$. hARID-5A = human ARID-5A (see Figure 1 for other definitions).

induced in RORγt-deficient mouse CD4+ T cells and WT mouse CD4+ T cells under Th17 cell–polarizing conditions (Figure 2F). These results indicate that the induction of ARID-5A depends on IL-6/STAT-3 signaling but not on RORγt, suggesting that Th17 cell differentiation is not needed for the induction of ARID-5A.

Forced expression of ARID-5A inhibits Th17 cell differentiation. Given that the expression of ARID-5A is enhanced in CD4+ T cells under Th17 cell–polarizing conditions (Figure 2), we next analyzed the effect of forced expression of ARID-5A in CD4+ T cells on Th17 cell differentiation. We used a bicistronic retrovirus-mediated gene expression system in which infected cells were identified by coexpressed green fluorescent protein (GFP). Purified naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 and infected with ARID-5A or control retrovirus under either Th0 cell conditions or Th17 cell–polarizing conditions. As shown in Figure 3, the forced expression of ARID-5A did not induce IL-17A (Figure 3A) or IL-17F (Figure 3B) under Th0 cell conditions. Importantly, under Th17 cell–polarizing conditions, IL-17A– or IL-17F–producing cells were significantly decreased by the forced expression of ARID-5A ($P < 0.05$) (n = 4 experiments) (Figures 3A and B). The forced expression of ARID-5A also significantly inhibited IL-22 production under Th22 cell–polarizing conditions (Figure 3C). Because the differentiation of Th17 cells and FoxP3+ Treg cells is counterbalanced (30), we next examined the effect of forced expression of ARID-5A on Treg cell differentiation. The expression of ARID-5A did not affect the induction of Treg cells under Th0 cell–, Th17 cell–, or Treg cell–polarizing conditions (further information is available at http://www.m.chiba-u.jp/class/allergy/). These results indicate that ARID-5A specifically inhibits Th17 and Th22 cell differentiation without affecting Treg cell differentiation.

Inhibition of RORγt-induced Th17 cell differentiation by ARID-5A. A previous study demonstrated that human ARID-5A interacted with nuclear hormone receptors, including estrogen receptor α (ERα), androgen receptor, retinoid X receptor α, and retinoic acid receptor (31). Because RORγt also belongs to the nuclear receptor superfamily, we next examined the effect of forced expression of ARID-5A on RORγt-induced Th17 cell differentiation. In this experiment, CD4+ T cells were doubly infected with GFP-expressing retroviruses (pMX-IRES-ARID5A-GFP or control pMX-IRES-GFP) and Thy1.1-expressing retroviruses (MSCV-myc-RORγt-IRES-Thy1.1 or control MSCV-IRES-Thy1.1) under Th0 cell conditions and analyzed for the expression of IL-17A in GFP+Thy1.1+ cells. The forced expression of ARID-5A significantly suppressed RORγt-induced IL-17A production in CD4+ T cells ($P < 0.01$)

(n = 4 experiments) (Figures 4A and B). Moreover, even in the presence of anti–IL-6 antibody to block possible intrinsic IL-6–induced Th17 cell differentiation, ARID-5A also significantly suppressed RORγt-induced IL-17A expression in CD4+ T cells (Figure 4B). These results suggest that ARID-5A may directly inhibit RORγt-induced IL-17A production in CD4+ T cells.

A recent study demonstrated that RORγt enhances the transcription of IL-17A by binding to the promoter region from −153 bp to −94 bp through

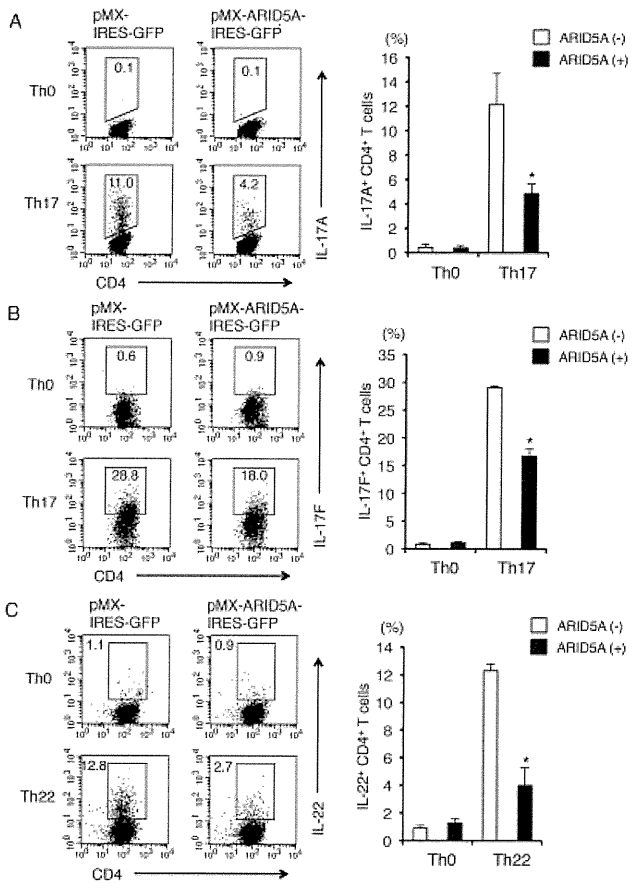

Figure 3. AT-rich–interactive domain–containing protein 5A (ARID-5A) inhibits Th17 cell differentiation. Murine naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions for 24 hours, and then cells were infected with a retrovirus of pMX-ARID5A-IRES-GFP (ARID-5A+) or pMX-IRES-GFP (ARID-5A−; as a control) under Th0 cell conditions, Th17 cell–polarizing conditions, or Th22 cell–polarizing conditions. Three days later, cells were restimulated with phorbol myristate acetate plus ionomycin for 5 hours, and intracellular cytokine profiles of interleukin-17A (IL-17A) (**A**), IL-17F (**B**), and IL-22 (**C**) in green fluorescent protein–positive CD4+ T cells were evaluated by fluorescence-activated cell sorting analysis. Left, Representative data of cytokine profiles in CD4+ T cells. Right, Frequency of IL-17A–, IL-17F–, or IL-22–producing CD4+ T cells. Values are the mean ± SD of 4 experiments. * = $P < 0.05$ versus control.

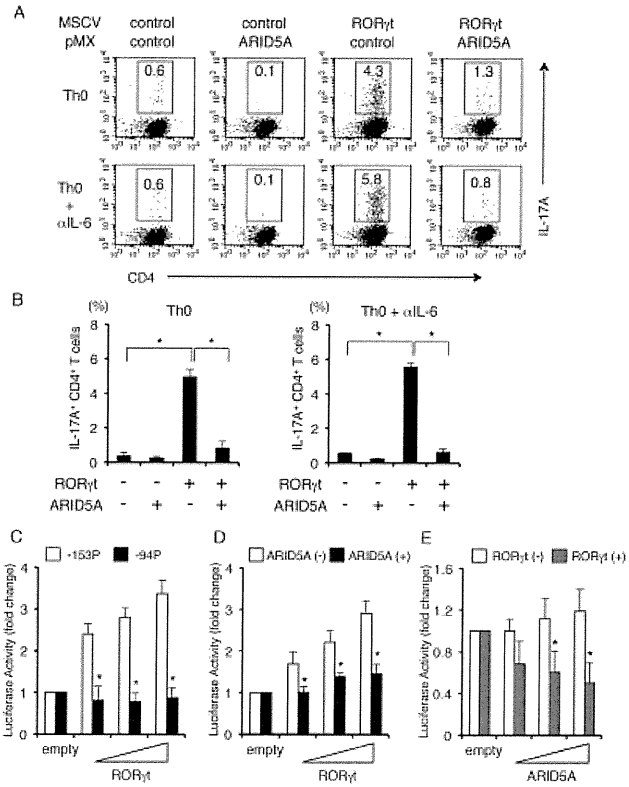

Figure 4. AT-rich–interactive domain–containing protein 5A (ARID-5A) inhibits retinoic acid receptor–related orphan nuclear receptor γt (RORγt)–induced Th17 cell differentiation. **A** and **B,** Murine naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions for 24 hours and then doubly infected with retroviruses of pMX-based virus (pMX-ARID5A-IRES-GFP or pMX-IRES-GFP) and MSCV-based virus (MSCV-myc-RORγt-IRES-Thy1.1 or MSCV-IRES-Thy1.1). Twenty-four hours later, cells were stimulated with anti-CD3/anti-CD28 for 3 days in the presence or absence of anti–interleukin-6 (anti–IL-6) antibody. Cells were restimulated with phorbol myristate acetate plus ionomycin for 5 hours, and the expression of IL-17A in doubly infected CD4+ T cells (GFP+Thy1.1+CD4+ cells) was evaluated. Shown are representative fluorescence-activated cell sorting profiles (**A**) and mean ± SD frequencies of IL-17A–producing CD4+ T cells (n = 4 experiments) (**B**). * = $P < 0.01$. **C,** EL4 cells were transfected with −153 mIL17p-Luc (−153P) or −94 mIL17p-Luc (−94P) in the presence of MSCV-myc-RORγt-IRES-Thy1.1 (0.25, 0.5, or 1 μg) or empty MSCV-IRES-Thy1.1. **D,** EL4 cells were transfected with −153 mIL17p-Luc in the presence of pcDNA3-ARID5A (ARID-5A+) or empty pcDNA3 (ARID-5A−) and various amounts (0.25, 0.5, or 1 μg) of MSCV-myc-RORγt-IRES-Thy1.1 or empty MSCV-IRES-Thy1.1. **E,** EL4 cells were transfected with −153 mIL17p-Luc in the presence of MSCV-myc-RORγt-IRES-Thy1.1 (RORγt+) or empty MSCV-IRES-Thy1.1 (RORγt−) and various amounts (0.25, 0.5, or 1 μg) of pcDNA3-ARID5A or empty pcDNA3. Values in **C–E** are the mean ± SD of 4 experiments. * = $P < 0.05$ versus control.

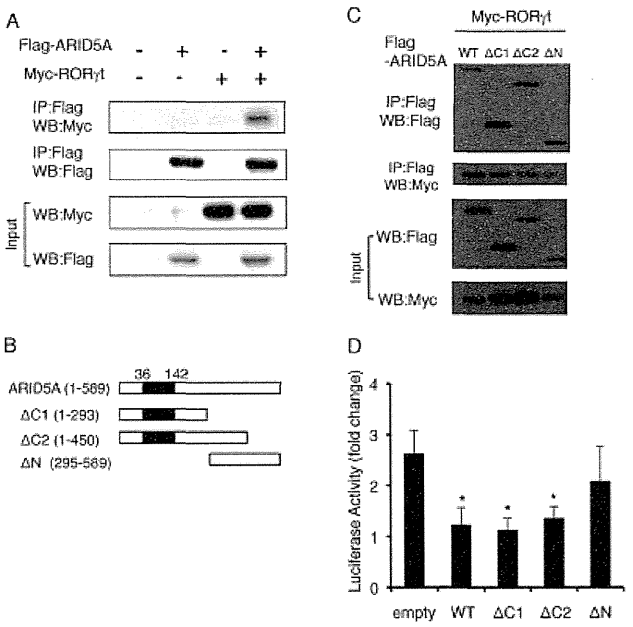

Figure 5. AT-rich–interactive domain–containing protein 5A (ARID-5A) physically associates with retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and inhibits its function. **A,** We cotransfected 293T cells with FLAG-tagged ARID-5A (pcDNA3-FLAG-ARID5A) and Myc-tagged RORγt (MSCV-myc-RORγt-IRES-Thy1.1). Whole cell lysate was immunoprecipitated (IP) with anti-FLAG monoclonal antibody (mAb) and subjected to Western blotting (WB) with anti-Myc mAb. Results are representative of 4 independent experiments. **B,** Shown is a schematic diagram of truncated mutants of ARID-5A. ΔC1 and ΔC2 indicate N-terminal amino acids 1–293 and 1–450, respectively, of ARID-5A. ΔN indicates C-terminal amino acid 295–589 of ARID-5A. **C,** We transfected 293T cells with FLAG-tagged wild-type (WT) or mutant ARID-5A and Myc-tagged RORγt. Coimmunoprecipitation assay was performed as described in **A. D,** EL4 cells were transfected with −153 mIL17p-Luc in the presence of MSCV-myc-RORγt-IRES-Thy1.1 (1 μg) and empty pcDNA3, pcDNA3-ARID5A (WT), or truncated mutants of ARID-5A. Twenty-four hours after transfection, the luciferase activity of reporter constructs was determined by dual luciferase assay. Values are the mean ± SD fold induction of luciferase activity relative to MSCV-myc-IRES-Thy1.1–transfected cells (n = 4 experiments). * = $P < 0.05$ versus empty pcDNA3–transfected cells.

retinoic acid–related orphan receptor response elements (19). We thus used reporter assays to examine the effect of ARID-5A on RORγt-induced activation of IL-17A promoter. We subcloned the truncated promoter regions (5′ to −153 bp and 5′ to −94 bp) into a luciferase reporter vector (termed −153 mIL17p-Luc and −94 mIL17p-Luc, respectively). Consistent with the previous study (19), the expression of RORγt enhanced the promoter activity of −153 mIL17p-Luc but not of −94 mIL17p-Luc in EL4 cells (Figure 4C). Cotransfection of ARID-5A with RORγt inhibited RORγt-induced activation of −153 mIL17p-Luc in a dose-dependent manner (Figures 4D and 4E). These results suggest that ARID-5A inhibits IL-17A production through the inhibition of RORγt-induced activation of IL-17A promoter.

ARID-5A associates with RORγt and suppresses its function. To determine whether ARID-5A physically associates with RORγt, we performed a coimmunoprecipitation assay. We transfected 293T cells with the expression vectors of FLAG-tagged ARID-5A and/or Myc-tagged RORγt, and we immunoprecipitated the cell lysates with anti-FLAG antibody, followed by immunoblotting with anti-Myc antibody. As shown in Figure 5A, the coimmunoprecipitation assay clearly demonstrated that ARID-5A physically associated with RORγt.

Finally, we investigated the functional domains of ARID-5A for the association with RORγt and the inhibition of RORγt-induced IL-17A induction. A coimmunoprecipitation assay with truncated mutants of ARID-5A (Figure 5B) revealed that the ΔC1 and ΔC2 mutants, but not the ΔN mutant, of ARID-5A bound to RORγt (Figure 5C). Consistent with these findings, reporter assays showed that the ΔC1 and ΔC2 mutants, but not the ΔN mutant, of ARID-5A significantly inhibited RORγt-induced activation of −153 mIL17p-Luc ($P < 0.05$) (n = 4 experiments) (Figure 5D). These results suggest that the N-terminal region of ARID-5A is required for the association with RORγt and the suppression of its function.

DISCUSSION

In this study, we showed that ARID-5A functions as a negative regulator of RORγt-induced Th17 cell differentiation. By analysis of gene expression profiles of CD4+ T cells in RA patients who exhibited good clinical responses to biologic therapies, we identified ARID-5A as a new molecule down-regulated by IL-6 blockade in the form of TCZ therapy (Table 1 and Figure 1B). We then found that IL-6 induced the expression of ARID-5A in CD4+ T cells during Th17 cell differentiation (Figures 2A–D) and that STAT-3 inhibitor VI inhibited the induction of ARID-5A in Th17 cells (Figure 2E). On the other hand, IL-6–induced expression of ARID-5A was normally observed in RORγt-deficient mouse CD4+ T cells (Figure 2F). Importantly, we also found that ARID-5A physically associated with RORγt (Figure 5) and inhibited RORγt-induced Th17 cell differentiation (Figure 4). Taken together, these results indicate that ARID-5A is a lineage-specific attenuator

of Th17 cell differentiation, suggesting that ARID-5A may be involved in the pathogenesis of RA.

We showed that ARID-5A is induced by IL-6 signaling in CD4+ T cells and inhibits Th17 cell differentiation. We found that IL-6 induced ARID-5A expression in murine CD4+ T cells and that the STAT-3 inhibitor suppressed IL-6–induced ARID-5A expression (Figure 2). On the other hand, IL-6–induced ARID-5A expression was normal in RORγt-deficient mouse CD4+ T cells (Figure 2), indicating that IL-6/STAT-3 signaling but not Th17 cell differentiation is involved in the induction of ARID-5A in CD4+ T cells. We also showed that forced expression of ARID-5A in CD4+ T cells inhibited IL-17A and IL-17F production by CD4+ T cells under Th17 cell–polarizing conditions (Figure 3) and also inhibited RORγt-induced Th17 cell differentiation even in the presence of a neutralizing antibody against IL-6 (Figure 4). On the other hand, ARID-5A did not significantly affect the differentiation of FoxP3+ Treg cells (further information is available at http://www.m.chiba-u.jp/class/allergy/), which suppress Th17 cell differentiation (30). Our findings thus suggest that RORγt is a molecular target of ARID-5A for the inhibition of Th17 cell differentiation.

ARID-5A is a member of the AT-rich–interactive domain family of nuclear proteins (32). It has been shown that ARID family members are involved in a wide range of biologic functions, including cell growth, differentiation, and development (33,34). A previous study has shown that ARID-5A interacts with ERα and suppresses ERα-induced transactivation (31). We show herein that ARID-5A associates with RORγt through its N-terminal region and suppresses the activity of RORγt and subsequent RORγt-induced Th17 cell differentiation (Figures 4 and 5). On the other hand, it has been demonstrated that ARID-5A interacts with SOX9 and enhances SOX9-induced chondrocyte-specific transcription (18). Therefore, it is suggested that ARID-5A could regulate the activity of its partners both positively and negatively depending on the interaction with its partners.

Over the last decade, a number of studies have revealed that not only Th1 cells, but also Th17 cells are involved in the pathogenesis of RA (6,7,35,36). The efficacy for RA of therapy with TCZ, an anti–IL-6 receptor mAb that antagonizes the effect of IL-6, which is required for Th17 cell differentiation (37), supports the notion that Th17 cell–mediated inflammatory responses are involved in the pathogenesis of RA. Consistent with this notion, a recent study has shown that secukinumab, a mAb against IL-17A, is efficacious in the treatment of RA (38). Our finding that the expression of Th17 cell–related genes, such as BATF, BCL3, and ARID5A, is decreased in RA patients who show good clinical responses to TCZ therapy (Table 1) also supports this notion.

In addition to RA, it has been demonstrated that Th17 cells are involved in the pathogenesis of other autoimmune diseases, including psoriasis (39), multiple sclerosis (40), and inflammatory bowel diseases (41). Because RORγt (the gene is RORC in humans) functions as a lineage-specifying transcription factor of Th17 cells, RORC seems to be a good therapeutic target in autoimmune diseases. Indeed, SR1001, an inverse agonist of RORα and RORγt, has been shown to reduce the severity of experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis (42). Digoxin and its derivatives have also been shown to suppress Th17 cell differentiation by antagonizing RORγt activity (43). Our findings of the inhibitory effect of ARID-5A on RORγt should provide an additional tool for the treatment of autoimmune diseases.

We found that the expression levels of ARID-5A in CD4+ T cells were elevated in untreated RA patients as compared with those in healthy controls (Figure 1A). Because Th17 cells have been shown to be increased in untreated RA patients (10) and because ARID-5A is induced in CD4+ T cells under Th17 cell–polarizing conditions (Figure 2), it is possible that the expression levels of ARID-5A in CD4+ T cells may also be increased in untreated RA patients in parallel with increased Th17 cells but may not be sufficient for the inhibition of RORγt-induced Th17 cell differentiation. On the other hand, it is also possible that the defective induction of ARID-5A in CD4+ T cells may cause increased Th17 cell differentiation in patients with RA. In this regard, we found that while the expression levels of ARID-5A under Th0 cell conditions were significantly higher in RA patients than in healthy controls, the enhanced induction of ARID-5A expression in CD4+ T cells was not significantly different between Th0 cell conditions and Th17 cell–polarizing conditions in RA patients (Figure 2A). Further analyses of the simultaneous measurement of ARID-5A and IL-17A at single CD4+ T cell levels is required to exclude the possibility that CD4+ T cells expressing IL-17A are different from those expressing ARID-5A under Th17 cell–polarizing conditions. In addition, the use of T cell–specific ARID-5A–deficient mice could enable us to elucidate the precise roles of ARID-5A in the pathogenesis of Th17 cell–mediated autoimmune diseases including RA.

Two RA patients did not respond to TCZ therapy

in this study. We found that CD4+ T cells from these 2 patients expressed levels of ARID-5A similar to those in RA patients who responded to TCZ therapy, but their ARID-5A levels were not significantly altered by TCZ therapy (data not shown). These results suggest that the down-regulation of ARID-5A expression is associated with the efficacy of TCZ therapy and may be a useful biomarker. A large-scale clinical study is needed to determine the value of the measurement of ARID-5A in clinical practice.

In conclusion, we have shown that ARID-5A, which is induced by IL-6/STAT-3 signaling in CD4+ T cells, physically associates with RORγt and inhibits RORγt-induced Th17 cell differentiation (further information is available at http://www.m.chiba-u.jp/class/allergy/). Although further studies are required, our results suggest that ARID-5A is a lineage-specific attenuator of Th17 cell differentiation and may have therapeutic potential for Th17 cell–mediated autoimmune diseases.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Kagami and Nakajima had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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