(Fig. 1B), although these patients showed good clinical responses to the therapy as evaluated by the CDAI (Table S2). These results suggest that the reduction of ARID5A expression in $CD4^+$ T cells is specifically associated with the efficacy of TCZ therapy but not with the reduced disease activity of RA itself.

In addition to ARID5A, we found that the signals of some IL-6-Stat3- 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 TNFi or ABT (Fig. S1). In contrast, the signals of some IL-6-Stat3- or Th17 cell-related genes including AHR and RORA were not significantly decreased in RA patients who were treated with TCZ, TNFi, or ABT (Fig. S1). On the other hand, the signals of other IL-6-Stat3- 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-Stat3 signaling induces the expression of ARID5A in Th17 cells**

To determine the roles of ARID5A in helper T cell differentiation, we examined the expression of ARID5A in human $CD4^+$ T cells stimulated with anti-CD3 mAb plus anti-CD28 mAb (anti-CD3/CD28) under various polarizing conditions. The expression of ARID5A was strongly induced in $CD4^+$ T cells from healthy controls under Th17-polarizing conditions (IL-6, IL-23, IL-1β, anti-IL-4, and anti-IFN-γ) as compared with non-polarizing Th0 conditions (Fig. 2A), whereas the expression of ARID5A was not significantly induced in Th1- or Th2-polarizing conditions (data not shown). Consistent with the enhanced expression of ARID5A in RA patients (Fig. 1A), the expression levels of ARID5A in $CD4^+$ T cells under Th0 conditions were significantly higher in RA patients than those in healthy controls (Fig. 2A). However, the induction of ARID5A expression in $CD4^+$ T cells was not significantly different between Th0 and Th17-polarizing conditions in RA patients (Fig. 2A).

We thereafter analyzed the roles of ARID5A in helper T cell differentiation by using

murine $CD4^+$ T cells. ARID5A was also highly expressed in murine $CD4^+$ T cells under Th17-polarizing conditions (IL-6, TGF-β, anti-IL-4, and anti-IFN-γ) as compared with Th0 conditions (Fig. 2B). The expression of ARID5A in Th17 cells was confirmed at protein levels by immunoblotting (Fig. 2C). Because ARID5A was induced under Th17-polarizing conditions, we next examined the effect of IL-6 and TGF-β, both of which are included in murine Th17-polarizing conditions, on the expression of ARID5A in $CD4^+$ T cells. As shown in Fig. 2D, IL-6 but not TGF-β significantly induced the expression of ARID5A in $CD4^+$ T cells ($n = 3$ experiments, $p<0.05$). These results suggest that ARID5A is induced in $CD4^+$ T cells by IL-6 under Th17-polarizing conditions.

To determine the signaling pathways that induce ARID5A expression under Th17-polarizing conditions, we then examined the effect of an inhibitor for Stat3 (Stat3 inhibitor VI) on ARID5A expression in $CD4^+$ T cells. As shown in Fig. 2E, Stat3 inhibitor VI partially but significantly suppressed ARID5A expression in $CD4^+$ T cells under Th17-polarizing conditions ($n = 3$ experiments, $p<0.05$). We also examined the role of RORγt, a lineage-specifying transcription factor of Th17 cells (13, 28), on ARID5A expression in $CD4^+$ T cells by using RORγt-deficient mice. Intriguingly, ARID5A was similarly induced in RORγt-deficient $CD4^+$ T cells and wild-type (WT) $CD4^+$ T cells under Th17-polarizing conditions (Fig. 2F). These results indicate that the induction of ARID5A depends on IL-6-Stat3 signaling but not on RORγt, suggesting that Th17 cell differentiation is dispensable for the induction of ARID5A.

**The enforced expression of ARID5A inhibits Th17 cell differentiation**

Given that the expression of ARID5A is enhanced in $CD4^+$ T cells under Th17-polarizing conditions (Fig. 2), we next analyzed the effect of enforced expression of ARID5A in $CD4^+$ T cells on Th17 cell differentiation. We employed a bicistronic retrovirus-mediated gene expression system in which infected cells were identified by

co-expressed GFP. Purified naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 and infected with ARID5A or control retrovirus in either Th0 or Th17-polarizing conditions. As shown in Fig. 3, the enforced expression of ARID5A did not induce IL-17A (Fig. 3A) or IL-17F (Fig. 3B) in Th0 conditions. Importantly, in Th17-polarizing conditions, IL-17A- or IL-17F-producing cells were significantly decreased by the enforced expression of ARID5A ($n = 4$ experiments, $p<0.05$) (Fig. 3A and 3B). The enforced expression of ARID5A also significantly inhibited IL-22 production in Th22-polarizing conditions (Fig. 3C). Because the differentiation of Th17 cells and $Foxp3^+$ regulatory T cells (Tregs) are counterbalanced (29), we next examined the effect of enforced expression of ARID5A on Treg differentiation. As shown in Fig. S2, the expression of ARID5A did not affect the induction of Tregs in Th0, Th17-, nor Treg-polarizing conditions. These results indicate that ARID5A specifically inhibits Th17 and Th22 cell differentiation without affecting Treg differentiation.

**ARID5A inhibits RORγt-induced Th17 cell differentiation**

A previous study demonstrated that human ARID5A interacted with nuclear hormone receptors including estrogen receptor α, androgen receptor, retinoid X receptor α, and retinoic acid receptor (30). Because RORγt also belongs to the nuclear receptor superfamily, we next examined the effect of enforced expression of ARID5A 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) in Th0 conditions, and analyzed for the expression of IL-17A in $GFP^+$ $Thy1.1^+$ cells. The enforced expression of ARID5A significantly suppressed RORγt-induced IL-17A production in $CD4^+$ T cells ($n = 4$ experiments, $p<0.01$) (Fig. 4A and 4B). Moreover, even in the presence of anti-IL-6 antibody to block possible intrinsic IL-6-induced Th17 cell

differentiation, ARID5A also significantly suppressed RORγt-induced IL-17A expression in $CD4^+$ T cells (Fig. 4B). These results suggest that ARID5A may directly inhibit RORγt-induced IL-17A production in $CD4^+$ T cells.

A recent study has demonstrated that RORγt enhances the transcription of IL-17A by binding to the promoter region from -153 to -94 through ROR-responsive elements (ROREs) (18). We thus examined the effect of ARID5A on RORγt-induced activation of IL-17A promoter by reporter assays. 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 (18), the expression of RORγt enhanced the promoter activity of -153 mIL17p-Luc but not of -94 mIL17p-Luc in EL4 cells (Fig. 4C). Co-transfection of ARID5A with RORγt inhibited RORγt-induced activation of -153 mIL17p-Luc in a dose-dependent manner (Fig. 4D and 4E). These results suggest that ARID5A inhibits IL-17A production through the inhibition of RORγt-induced activation of IL-17A promoter.

**ARID5A associates with RORγt and inhibits its function**

To determine whether ARID5A physically associates with RORγt, we next performed co-immnoprecipitation assay. 293T cells were transfected with the expression vectors of Flag-tagged ARID5A and/or Myc-tagged RORγt, and the cell lysates were immunoprecipitated with anti-Flag antibody, followed by immunoblotting with anti-Myc antibody. As shown in Fig. 5A, co-immnoprecipitation assay clearly showed that ARID5A physically associated with RORγt.

We finally investigated the functional domains of ARID5A for the association with RORγt and the inhibition of RORγt-induced IL-17A induction. Co-immnoprecipitation assay with truncated mutants of ARID5A (Fig. 5B) revealed that ΔC1 and ΔC2 mutants but not ΔN mutant of ARID5A bound to RORγt (Fig. 5C). Consistently, reporter assays showed that ΔC1 and ΔC2 mutants but not ΔN mutant of ARID5A significantly

inhibited RORγt-induced activation of -153 mIL17p-Luc (n = 4 experiments, $p<0.05$) (Fig. 5D). These results suggest that N-terminal region of ARID5A is required for the association with RORγt and the suppression of its function.

## DISCUSSION

In this study, we show that ARID5A functions as a negative regulator of RORγt-induced Th17 cell differentiation. By the analysis of gene expression profiles of $CD4^+$ T cells in RA patients who exhibited good clinical responses to the biologic therapies, we identified ARID5A as a new molecule downregulated by IL-6 blockade by TCZ therapy (Table 1 and Fig. 1B). We then found that IL-6 induced the expression of ARID5A in $CD4^+$ T cells during Th17 cell differentiation (Fig. 2A-2D) and that Stat3 inhibitor VI inhibited the induction of ARID5A in Th17 cells (Fig. 2E). On the other hand, IL-6-induced expression of ARID5A was normally observed in RORγt-deficient $CD4^+$ T cells (Fig. 2F). Importantly, we also found that ARID5A physically associated with RORγt (Fig. 5) and inhibited RORγt-induced Th17 cell differentiation (Fig. 4). Taken together, these results indicate that ARID5A is a lineage-specific attenuator of Th17 cell differentiation, suggesting that ARID5A may be involved in the pathogenesis of RA.

We show that ARID5A is induced by IL-6 signaling in $CD4^+$ T cells and inhibits Th17 cell differentiation. We found that IL-6 induced ARID5A expression in murine $CD4^+$ T cells and the Stat3 inhibitor suppressed IL-6-induced ARID5A expression (Fig. 2). On the other hand, IL-6-induced ARID5A expression was normal in RORγt-deficient $CD4^+$ T cells (Fig. 2), indicating that IL-6-Stat3 signaling but not Th17 cell differentiation is involved in the induction of ARID5A in $CD4^+$ T cells. We also showed that the enforced expression of ARID5A in $CD4^+$ T cells inhibited IL-17A and IL-17F production of $CD4^+$ T cells in Th17-polarizing conditions (Fig. 3) and also inhibited RORγt-induced Th17 cell differentiation even in the presence of a neutralizing antibody against IL-6 (Fig. 4). On the other hand, ARID5A did not significantly affect the differentiation of $Foxp3^+$ Tregs (Fig. S2), which suppress Th17 cell differentiation (29). Our findings thus suggest that RORγt is a molecular target of ARID5A for the

inhibition of Th17 cell differentiation.

ARID5A is a member of ARID (AT-rich interaction domain) family of nuclear proteins (31). It has been shown that ARID family members are involved in a wide range of biological functions including cell growth, differentiation, and development (32, 33). A previous study has shown that ARID5A interacts with estrogen receptor (ER) α and suppresses ERα-induced transactivation (30). We show that ARID5A associates with RORγt through its N-terminal region and suppresses the activity of RORγt and subsequent RORγt-induced Th17 cell differentiation (Fig. 4 and Fig. 5). On the other hand, it has been demonstrated that ARID5A interacts with Sox9 and enhances Sox9-induced chondrocyte-specific transcription (17). Therefore, it is suggested that ARID5A could regulate the activity of its partners both positively and negatively depending on the interaction with its partners.

Over the past 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, 34, 35). The efficacy for RA of TCZ, an anti-IL-6 receptor monoclonal antibody which antagonizes the effect of IL-6 that is required for Th17 cell differentiation (36), supports the notion that Th17 cell-mediated inflammatory responses are involved in the pathogenesis of RA. In agreement with this notion, a recent study has shown that secukinemab, a monoclonal antibody against IL-17A, is efficacious for RA (37). Our finding that the expression of Th17 cell-related genes such as BATF, BCL3, and herein 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 (38), multiple sclerosis (39), and inflammatory bowel diseases (40). Because RORγt (RORC in humans) functions as a lineage-specifying transcription factor of Th17 cells, RORC seems a good therapeutic target of autoimmune diseases. Indeed, SR1001, an inverse agonist of

RORα and RORγt, has been shown to reduce the severity of experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (41). Digoxin and its derivatives have also been shown to suppress Th17 cell differentiation by antagonizing RORγt activity (42). Our findings of the inhibitory function of ARID5A against RORγt should provide an additional tool for the treatment of autoimmune diseases.

We found that the expression levels of ARID5A in $CD4^+$ T cells were elevated in untreated RA patients as compared with those in healthy controls (Fig. 1A). Because Th17 cells have been shown to be increased in untreated RA patients (10) and because ARID5A is induced in $CD4^+$ T cells under Th17-polarizing conditions (Fig. 2), it is possible that the expression levels of ARID5A 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 RORC-induced Th17 cell differentiation. On the other hand, it is also possible that the defective induction of ARID5A 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 ARID5A in Th0 conditions were significantly higher in RA patients than those in healthy controls, the enhanced induction of ARID5A expression in $CD4^+$ T cells was not significantly different between Th0 and Th17-polarizing conditions in RA patients (Fig. 2A). Further analyses of simultaneous measurement of ARID5A 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 ARID5A under Th17-polarizing conditions. In addition, T cell-specific ARID5A-deficient mice could elucidate the precise roles of ARID5A 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 of the patients expressed similar levels of ARID5A to those of RA patients who responded to TCZ therapy, but the levels of ARID5A were not significantly altered by TCZ therapy in the patients (data not shown). These results suggest that the

down-regulation of ARID5A expression is associated with the efficacy of TCZ therapy and may be useful for a biomarker. A large-scale clinical study is needed to determine the value of the measurement of ARID5A in the clinical practice of RA.

In conclusion, we have shown that ARID5A, which is induced by IL-6-Stat3 signaling in $CD4^+$ T cells, physically associates with RORγt and inhibits RORγt-induced Th17 cell differentiation (Fig. S3). Although further studies are required, our results suggest that ARID5A is a lineage-specific attenuator of Th17 cell differentiation and may have a therapeutic potential for Th17 cell-mediated autoimmune diseases.

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**TABLE**

**Table 1. WAD ranking of differentially expressed genes in $CD4^+$ T cells from RA patients who respond to Tocilizumab therapy**

| WAD Rank | Gene Name | Regulation (0w to 12w) | Fold Change Absolute | 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 |

WAD, Weighted Average Difference

## FIGURE LEGENDS

**Figure 1. The expression of ARID5A in $CD4^+$ T cells from RA patients is decreased by Tocilizumab 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. *p<0.01. (**B**) $CD4^+$ T cells were isolated from RA patients who showed good clinical responses to the treatment with Tocilizumab (n = 8), TNF inhibitors (n = 13), or Abatacept (n = 12) at just before and 12 weeks after the treatment. Samples were subjected to DNA microarray analysis and array signals of ARID5A before and after the treatment were compared in each treatment group. *p<0.01. NS = not significant.

**Figure 2. IL-6-Stat3 signaling induces ARID5A 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/CD28 in Th0 or Th17-polarizing conditions and the expression of ARID5A was measured by qPCR. *p<0.05. (**B**) Murine naïve $CD4^+$ T cells were stimulated in Th0 or Th17-polarizing conditions for indicated time periods and the expression of ARID5A was measured by qPCR. Data are representative of three independent experiments. (**C**) Naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 in Th0 or Th17-polarizing conditions and were subjected to immunoblotting with anti-ARID5A antibody. As controls, lysates of 293T cells that were transfected with pcDNA3-ARID5A or empty pcDNA3 were used. (**D-F**) (**D**) Naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 in the presence or absence of IL-6 and/or TGF-β. (**E**) Naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 in Th0 or Th17-polarizing conditions in the presence or absence of Stat3 inhibitor VI. (**F**) Naïve $CD4^+$ T cells from RORγt-deficient mice or littermate wild-type (WT) mice were stimulated with

anti-CD3/CD28 in Th0 or Th17-polarizing conditions. ARID5A expression was measured by qPCR. Data are means ± SD from three experiments. *p<0.05.

**Figure 3. ARID5A inhibits Th17 cell differentiation**

(**A-C**) Murine naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 in Th0 conditions for 24 h and then cells were infected with a retrovirus of pMX-ARID5A-IRES-GFP or pMX-IRES-GFP (as a control) in Th0, Th17-, or Th22-polarizing conditions. Three days later, cells were re-stimulated with PMA plus ionomycin for 5 h and intracellular cytokine profiles of IL-17A (**A**), IL-17F (**B**), and IL-22 (**C**) in $GFP^+$ $CD4^+$ T cells were evaluated by FACS analysis. Left panels show representative data of cytokine profiles in $CD4^+$ T cells, and right panels show the frequency of IL-17A, IL-17F, or IL-22-producing $CD4^+$ T cells. Data are means ± SD from four experiments. *, significantly different from the mean value of the corresponding control response, p<0.05.

**Figure 4. ARID5A inhibits RORγt-induced Th17 cell differentiation**

(**A-B**) Murine naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 in Th0 conditions for 24 h 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/CD28 for 3 days in the presence or absence of anti-IL-6 antibody. Cells were re-stimulated with PMA+ionomycin for 5 h and the expression of IL-17A in doubly-infected $CD4^+$ T cells ($GFP^+$ $Thy1.1^+$ $CD4^+$ cells) was evaluated. Shown are representative FACS profiles (**A**) and means ± SD of the frequency of IL-17A-producing $CD4^+$ T cells (**B**). n = 4 experiments. *p<0.01. (**C**) EL4 cells were transfected with -153 mIL17p-Luc or -94 mIL17p-Luc in the presence of MSCV-myc-RORγt-IRES-Thy1.1 (0.25, 0.5, or 1 μg) or empty

MSCV-myc-IRES-Thy1.1. (**D**) EL4 cells were transfected with -153 mIL17p-Luc in the presence of pcDNA3-ARID5A or empty pcDNA3 and various amounts (0.25, 0.5, or 1 μg) of MSCV-myc-RORγt-IRES-Thy1.1. (**E**) EL4 cells were transfected with -153 mIL17p-Luc in the presence of MSCV-myc-RORγt-IRES-Thy1.1 or empty MSCV-myc-IRES-Thy1.1 and various amounts (0.25, 0.5, or 1 μg) of pcDNA3-ARID5A. Data are means ± SD. n = 4 experiments. *, $p<0.05$.

**Figure 5. ARID5A physically associates with RORγt and inhibits its function**

(**A**) 293T cells were co-transfected with Flag-tagged ARID5A (pcDNA3-Flag-ARID5A) and Myc-tagged RORγt (MSCV-myc-RORγt-IRES-Thy1.1). Whole cell lysate was immunoprecipitated with anti-Flag mAb and blotted with anti-Myc mAb. Shown are representative of 4 independent experiments. (**B**) A schematic diagram of truncated mutants of ARID5A. ΔC1 and ΔC2 indicate N-terminal amino acid 1-293 and 1-450 of ARID5A, respectively. ΔN indicates C-terminal amino acid 295-589 of ARID5A. (**C**) 293T cells were transfected with Flag-tagged wild-type (WT) or mutant ARID5A and Myc-tagged RORγt. Co-immunoprecipitation 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 its truncated mutants. Twenty-four hours after the transfection, the luciferase activity of reporter constructs was determined by dual luciferase assay. Data are means ± SD of fold induction of luciferase activity relative to MSCV-myc-IRES-Thy1.1-transfected cells. n = 4 experiments. *, significantly different from the mean value of the empty pcDNA3-transfected cells, $p<0.05$.

## SUPPLEMENTAL FIGURE LEGEND

**Figure S1. The expression of Th17 signature genes in $CD4^+$ T cells from RA patients treated by Tocilizumab, TNF inhibitors, or Abatacept**

$CD4^+$ T cells were isolated from RA patients who showed good clinical responses to the treatment with Tocilizumab (TCZ) (n = 8), TNF inhibitors (TNFi) (n = 13), or Abatacept (ABT) (n = 12) at just before and 12 weeks after the treatment as described in Fig. 1B. Samples were subjected to DNA microarray analysis and array signals of BATF, NFKBIZ, BCL3, AHR, MAF, RORA, SOCS3, SOCS1, and ICOS before and after the treatment were compared in each treatment group. *$p<0.05$.

**Figure S2. ARID5A does not affect Treg differentiation**

Murine naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 in Th0 conditions for 24 h and then infected with a retrovirus of pMX-ARID5A-IRES-GFP or pMX-IRES-GFP (as a control) in Th0, Th17-, or Treg-polarizing conditions. Three days later, intracellular staining for Foxp3 was performed similarly as in Figure 3. Shown are representative Foxp3 staining of infected $GFP^+$ $CD4^+$ T cells from 4 independent experiments.

**Figure S3. Schematic diagram of the action of ARID5A on RORγt-induced Th17 cell differentiation**

IL-6 induces the expression of ARID5A in $CD4^+$ T cells during Th17 cell differentiation by a Stat3-dependent mechanism. ARID5A physically associates with RORγt by its N-terminal region and inhibits RORγt-induced IL-17A expression.