by the manufacturer (Applied Biosystems). Data were normalized to expression levels of GAPDH and/or ubiquitin C.

Statistical analysis. Statistical analysis was performed using SPSS version 21.0 (IBM Japan). Normally distributed continuous data were expressed as the mean ± SD and were analyzed using parametric tests (2-sample *t*-test [Welch's *t*-test when 2 variances were not considered equal] or paired *t*-test). Non-normally distributed data were expressed as the median and interquartile range and were analyzed using nonparametric tests (Mann-Whitney U test). Multivariate analyses were performed using logistic regression models. *P* values less than 0.05 were considered significant.

RESULTS

Patients and disease characteristics. Characteristics of patients in both the training cohort and validation cohort are shown in Table 1. All patients were Japanese; the mean age was 58.4 years and 60.9 years in the training and validation cohorts, respectively. The training cohort was composed of 77.5% women, and the validation cohort was composed of 80.0% women. The median disease duration was 57.5 months and 44.5 months in the 2 cohorts, respectively. All patients fulfilled the 2010 ACR/EULAR criteria for RA (22) in addition to the ACR 1987 revised criteria. Methotrexate was administered to 72.5% of patients in the training cohort and 75.0% of the patients in the validation cohort (median weekly dosage 8 mg and 6.75 mg, respectively), and corticosteroids were administered to 57.5% of patients in the training cohort and 55.0% of patients in the validation cohort (median daily dosage of prednisolone 3.875 mg and 1 mg, respectively). TNF antagonists had been administered to 62.5% of patients in the training cohort and 65.0% of patients in the validation cohort. One patient in the training cohort had received rituximab as part of the treatment regimen for malignant lymphoma 4 years before commencing TCZ treatment. No other biologic agents had been administered previously.

Thirteen healthy donors were also enrolled in this study. The mean ± SD age was 47.5 ± 8.3 years, and 10 of the patients (76.9%) were women.

Response to TCZ treatment. Based on physician's global assessment, a good or moderate response to TCZ treatment was achieved in 29 patients in the training cohort at 6 months, while 8 patients did not respond. Three patients were excluded from further analyses because TCZ was discontinued in those patients before they had received 3 months of treatment, due to either an acute exacerbation of cervical spondylosis, necessitating surgery (n = 1) or poor patient compliance with the scheduled visits for TCZ administration (n = 2).

In the validation cohort, all patients were eligible for analysis. A good or moderate response was achieved in 15 patients, whereas no response was observed in 5 patients.

Significantly or numerically larger improvement in disease activity measures was seen in patients who had been classified as responders by physician's global assessment as compared with those who had been classified as nonresponders, although the differences were less significant in the validation cohort due to the small sample size (Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://online

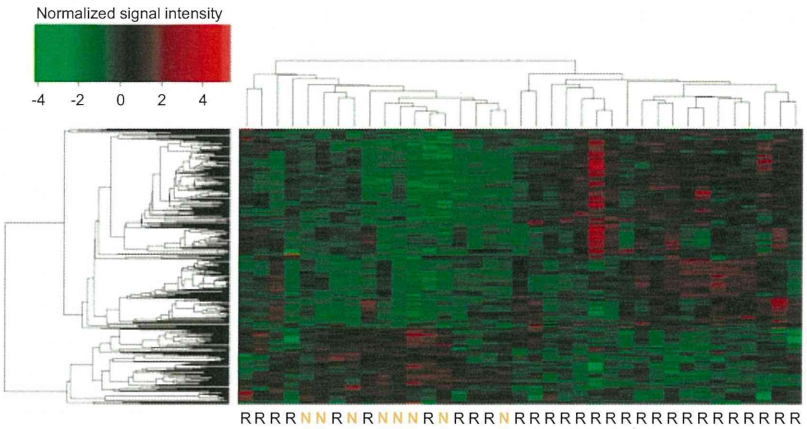

Figure 1. Hierarchical clustering of signal intensity patterns for individual patients and DNA microarray probes, identified by comparing those who responded to tocilizumab treatment with those who did not respond to tocilizumab treatment in the training cohort. The heatmap shows the normalized signal intensities of 409 probes derived from the genes of 37 patients (29 responders [R] and 8 nonresponders [N]) in the training cohort.

Table 2. Candidate DNA microarray probes identified by comparisons between nonresponders and responders in the training cohort*

Gene, DNA microarray probe	Difference in normalized signal intensity					Correlation with qPCR expression levels‡	
	Log_2-transformed signal intensity, mean ± SD		Absolute fold difference	Up-/down-regulation in responders	P†	r	P§
	Nonresponders	Responders					
CCL3L3							
A_23_P321920	0.753 ± 1.034	1.987 ± 1.873	2.4	Up	0.023	0.994	<0.001
A_24_P228130	0.871 ± 1.054	2.064 ± 1.826	2.3	Up	0.028	0.998	<0.001
CCL4, A_23_P207564	3.394 ± 0.846	4.251 ± 1.138	1.8	Up	0.034	0.859	0.001
CD83, A_23_P70670	2.450 ± 1.257	3.718 ± 1.489	2.4	Up	0.031	0.858	0.001
CXCR4, A_23_P102000	6.331 ± 0.430	7.032 ± 0.674	1.6	Up	0.002	0.839	0.002
FOSL2, A_23_P218555	−0.352 ± 0.780	0.526 ± 1.017	1.8	Up	0.020	0.868	0.001
HP, A_23_P206760	4.187 ± 0.890	3.204 ± 1.485	2.0	Down	0.030	0.839	0.002
HPR, A_23_P421493	0.262 ± 0.970	-0.710 ± 1.496	2.0	Down	0.041	0.623	0.055
IFI6, A_23_P201459	1.755 ± 0.489	2.698 ± 1.232	1.9	Up	0.003	0.633	0.049
IL27, A_23_P315320	2.216 ± 0.765	1.193 ± 1.553	2.0	Down	0.016	0.230	0.620
LY6E, A_24_P317762	−0.300 ± 0.403	0.437 ± 0.956	1.7	Up	0.003	0.671	0.048
MT1B, A_23_P37983	1.956 ± 0.427	2.601 ± 0.776	1.6	Up	0.006	ND	ND
MT1G, A_23_P60933	2.293 ± 0.402	2.923 ± 0.795	1.5	Up	0.005	0.663	0.037
MT1L, A_23_P427703	2.091 ± 0.383	2.689 ± 0.772	1.5	Up	0.006	0.482	0.158
MT2A							
A_23_P106844	4.476 ± 0.411	5.120 ± 0.867	1.6	Up	0.006	0.672	0.033
A_23_P252413	4.012 ± 0.455	4.624 ± 0.908	1.5	Up	0.015	0.663	0.037
A_24_P361896	4.246 ± 0.427	4.969 ± 0.813	1.7	Up	0.003	0.787	0.007
MX2, A_24_P117294	0.767 ± 0.340	1.410 ± 0.912	1.6	Up	0.004	0.861	0.001
OASL, A_23_P139786	1.407 ± 0.640	2.190 ± 1.255	1.7	Up	0.024	0.942	<0.001
RABGEF1							
A_23_P250825	−0.229 ± 0.675	0.425 ± 1.038	1.6	Up	0.048	0.923	<0.001
A_24_P232049	−0.456 ± 0.723	0.234 ± 1.045	1.6	Up	0.047	0.920	<0.001
THBS1, A_24_P142118	−0.341 ± 1.349	1.088 ± 1.732	2.7	Up	0.026	0.841	0.002
WARS, A_23_P65651	2.685 ± 0.389	3.355 ± 0.642	1.6	Up	0.002	0.897	<0.001

* Candidate genes are listed in alphabetical order. ND = not determined.
† By 2-sample *t*-test.
‡ Expression levels of GAPDH were used to normalize the quantitative polymerase chain reaction (qPCR) data.
§ By Pearson's correlation coefficient.

library.wiley.com/doi/10.1002/art.38400/abstract). The CDAI category improved (e.g., from moderate disease activity to low disease activity) in 28 of 29 responders in the training cohort and in all 15 responders in the validation cohort.

Differences in patient and disease characteristics between nonresponders and responders. As shown in Table 1, no significant differences in baseline characteristics between nonresponders and responders were identified in either the training cohort or the validation cohort.

Identification of candidate genes. Signal intensity values of 41,000 probes for 19,416 genes in 8 nonresponders and 29 responders were obtained. First, we excluded 15,564 probes for 5,755 genes with a signal intensity that was at a background level in all specimens (<100 relative fluorescence units). We then identified 409 probes that fulfilled the following conditions: $P < 0.05$ by 2-sample *t*-test (for the difference in normalized signal intensities between nonresponders and responders), and a fold difference of >1.5 in normalized signal intensities between nonresponders and responders. Figure 1 shows a heatmap of normalized signal intensities and the hierarchical clustering analyses of these 409 probes. Gene expression patterns for nonresponders clustered in the same branch, suggesting that a set of these genes can be a sensitive biomarker for the identification of patients whose RA is not likely to improve with TCZ treatment.

We further narrowed the pool of candidates to 68 probes by applying the following conditions: $P < 0.05$ by 2-sample *t*-test (for the difference in normalized signal intensities between nonresponders and responders as determined by the change in CDAI category), a fold difference of >1.5 in normalized signal intensities between nonresponders and responders as determined by the change in CDAI category, and a mean normalized signal intensity >0 (log_2 scale) among either nonresponders or responders.

We chose 23 probes that represented 19 genes

Table 3. Differences in baseline relative expression of candidate genes between nonresponders and responders in the validation cohort*

Gene	Expression relative to GAPDH, mean ± SD			Expression relative to ubiquitin C, mean ± SD		
	Nonresponders	Responders	*P*	Nonresponders	Responders	*P*
CCL3L3	0.22908 ± 0.11719	0.71191 ± 1.14197	0.128	0.13394 ± 0.08251	0.41982 ± 0.82582	0.207
CCL4	0.47432 ± 0.52809	0.32468 ± 0.16105	0.565	0.32773 ± 0.46202	0.18158 ± 0.09879	0.520
CD83	0.09453 ± 0.06540	0.12588 ± 0.20742	0.623	0.11224 ± 0.11309	0.08877 ± 0.14512	0.721
CXCR4	2.02442 ± 0.52763	2.60166 ± 1.34114	0.186	2.51513 ± 1.10834	2.18558 ± 1.03989	0.578
FOSL2	0.12600 ± 0.05244	0.15703 ± 0.13811	0.477	0.06256 ± 0.01640	0.08387 ± 0.10172	0.446
HP	0.01959 ± 0.01192	0.03599 ± 0.03659	0.148	0.00705 ± 0.00403	0.01579 ± 0.01562	0.064
IFI6	0.01166 ± 0.00514	0.01517 ± 0.01106	0.038	0.01982 ± 0.00674	0.07001 ± 0.08707	0.043
LY6E	0.21193 ± 0.09510	0.41288 ± 0.45754	0.128	0.17700 ± 0.07236	0.43286 ± 0.75126	0.213
MT1G	0.00039 ± 0.00030	0.00164 ± 0.00128	0.003	0.00050 ± 0.00040	0.00150 ± 0.00162	0.041
MT2A	0.26977 ± 0.10763	0.35474 ± 0.24362	0.299	0.14168 ± 0.08079	0.16255 ± 0.08920	0.640
MX2	0.07054 ± 0.02718	0.13847 ± 0.08220	0.012	0.04406 ± 0.01432	0.11390 ± 0.11756	0.039
OASL	0.03208 ± 0.00883	0.07313 ± 0.06817	0.038	0.01172 ± 0.00470	0.02848 ± 0.02597	0.029
RABGEF1	0.03439 ± 0.01607	0.05279 ± 0.03053	0.107	0.02816 ± 0.01219	0.05486 ± 0.05447	0.094
THBS1	0.12593 ± 0.10264	0.24968 ± 0.26263	0.149	0.07945 ± 0.07671	0.15341 ± 0.17254	0.207
WARS	0.42023 ± 0.15457	0.51446 ± 0.28856	0.371	0.27259 ± 0.13079	0.27193 ± 0.12630	0.992

* Candidate genes are listed in alphabetical order. GAPDH and ubiquitin C were used as internal controls to normalize data. *P* values were determined by 2-sample *t*-test.

(Table 2). These genes were selected based on fulfillment of any of the following criteria: the gene had multiple probes (e.g., *RABGEF1*), the gene was one of a group of genes that belonged to the same family (e.g., *MT1B, MT1G, MT1L,* and *MT2A*), or the gene was directly involved in the immune/inflammatory response (e.g., *IL27*).

Correlation between DNA microarray signal intensity and relative expression determined using qPCR analysis. Expression levels of the 19 genes (in 10 randomly selected complementary DNA samples) were determined by qPCR analysis using GAPDH to normalize the data, and the relative expression was compared with the DNA microarray signal intensity of the same sample in order to exclude the genes that had expression that was not likely to be reproduced. A meaningful amplification curve was not obtained for *MT1B* using any set of primers. Of the remaining 18 genes, statistically significant correlation between DNA microarray signal intensity and relative expression (as determined by qPCR analysis) was confirmed for 15 genes (Table 2).

Validation of differential gene expression between nonresponders and responders in an independent cohort. Differences in expression levels of these genes in PBMCs were compared between nonresponders and responders in the validation cohort. Supplementary Figure 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38400/abstract) shows a heatmap of the relative expression levels (using GAPDH as an internal control) and clustering of their patterns. The gene expression patterns for nonresponders clustered in the same branch, suggesting that a combination of these genes can be a sensitive biomarker for use in identifying patients whose RA is not likely to improve with TCZ treatment. However, significantly higher expression levels in responders were reproduced only in 4 genes (*IFI6*, *MT1G*, *MX2*, and *OASL*), and similar results were obtained when ubiquitin C was used as an internal control (Table 3).

Comparisons between healthy controls and RA patients and between patients before and after TCZ treatment. Normalized DNA microarray signal intensities of all 4 genes identified were significantly higher in RA patients who responded to TCZ than in healthy controls (Figure 2). In addition, normalized signal intensities tended to decrease after 3 months of TCZ treatment in responders but not in nonresponders (Figure 2). These data indicate that the expression of these genes in PBMCs is preferentially increased in patients with active RA who are likely to respond to TCZ treatment.

Prediction models for clinical responses to TCZ treatment. To assess the predictive values and determine optimal cutoff levels, we analyzed the 4 identified genes using a receiver operating characteristic (ROC) curve. For the prediction of moderate-to-good responses to TCZ treatment in the validation cohort, ROC analysis showed that the area under the curve (AUC) was 0.693

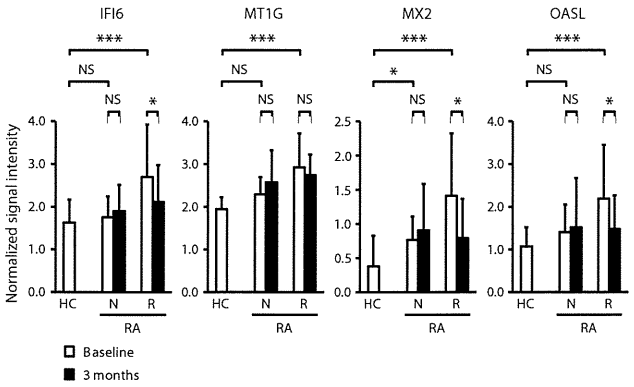

Figure 2. Comparisons of DNA microarray signal intensities between healthy controls (HCs), rheumatoid arthritis (RA) patients who were classified as nonresponders (N), and RA patients who were classified as responders (R), and comparisons of DNA microarray signal intensities within each group between baseline and after 3 months of tocilizumab treatment. DNA microarray signal intensities were determined for *IFI6*, *MT1G*, *MX2*, and *OASL*. Values are the mean ± SD. * = $P < 0.05$; *** = $P < 0.001$, by 2-sample *t*-test or paired *t*-test. NS = not significant.

for *IFI6*, 0.920 for *MT1G*, 0.813 for *MX2*, and 0.627 for *OASL* (Table 4).

We next performed multivariate logistic regression analyses to determine the independent predictive values of the identified genes that were associated with a moderate-to-good response to TCZ in the validation cohort. However, neither continuous nor dichotomous variables were identified as significant predictors (probably due to the small sample size).

We therefore assigned 1 point to each gene when the relative expression was above the cutoff point and calculated the total scores by summing these points. The predictive values of the total scores (with all possible combinations of the 4 genes) are shown in Table 4. An AUC of 0.947 (the largest of the AUCs) at a cutoff point of ≥2 was seen when total scores included the genes *MT1G* and *MX2* or the genes *MT1G*, *MX2*, and *OASL*. Positive and negative predictive values of these models for a moderate-to-good response to TCZ in the validation cohort were 100% and 55.6%, respectively.

DISCUSSION

This study is the first to identify, using human genome-wide DNA microarray analysis, candidate biomarkers that can be used to predict therapeutic responses to TCZ in patients with RA. Of 19,416 genes examined, 4 genes were identified as predictive biomarkers using data from 2 independent cohorts. Models combining these genes provided good predictive values for therapeutic responses to TCZ.

Table 4. ROC analyses and diagnostic values for each gene and for total scores for prediction of moderate-to-good responses to tocilizumab in the validation cohort*

Gene	ROC analysis		Diagnostic value, %			
	AUC	Optimal cutoff point	Sensitivity	Specificity	PPV	NPV
Single gene						
IFI6	0.693	≥0.85295	80	60	86	50
MT1G	0.920	≥0.00054	87	80	93	67
MX2	0.813	≥0.06587	87	80	93	67
OASL	0.627	≥0.04068	47	100	100	39
Total score						
IFI6/MT1G	0.887	≥2	73	100	100	56
IFI6/MX2	0.807	≥2	80	80	92	57
IFI6/OASL	0.793	≥1	80	60	86	50
MT1G/MX2	0.947	≥2	73	100	100	56
MT1G/OASL	0.880	≥1	87	80	93	67
MX2/OASL	0.880	≥1	87	80	93	67
IFI6/MT1G/MX2	0.913	≥3	73	100	100	56
IFI6/MT1G/OASL	0.887	≥2	73	100	100	56
IFI6/MX2/OASL	0.853	≥2	80	80	92	57
MT1G/MX2/OASL	0.947	≥2	73	100	100	56
IFI6/MT1G/MX2/OASL	0.913	≥3	73	100	100	56

* Receiver operating characteristic (ROC) analysis was performed for the relative expression level of each gene, using GAPDH as an internal control. ROC analysis was further performed for the total scores of all possible gene combinations. AUC = area under the curve; PPV = positive predictive value; NPV = negative predictive value.

Among the 4 genes identified in this study, *IFI6* (interferon-α [IFNα]–inducible protein 6), *MX2* (myxovirus resistance 2), and *OASL* (2′-5′-oligoadenylate synthetase–like gene) were type I IFN response genes (genes for which expression is induced by type I IFN signaling). Their increased expression was associated with favorable therapeutic responses to TCZ. Because responders did not have elevated expression levels of type I IFNs in PBMCs (data not shown), the major producer of the cytokines responsible for the increased expression of IFN response genes in responders seems to be other cell populations.

Type I IFNs, which consist of IFNα and IFNβ, are ubiquitously expressed in various cell types and have an essential function in mediating innate immune responses against viruses; they play critical roles in several immunologic processes including lymphoid differentiation, homeostasis, tolerance, and memory (23). It has been reported that the activity of type I IFN and the expression of IFN response genes in peripheral blood samples are increased in patients with RA (24–29). Mavragani et al demonstrated that the increased activity of IFN in plasma was a predictor of good clinical response in RA patients treated with TNF antagonists (25), and in a study by van Baarsen et al, the expression of some IFN response genes was increased in RA patients who exhibited a favorable response (30). In contrast, other studies have shown that increased expression of IFN response genes and IFNα in peripheral blood cells (13,26) or synovial tissue (31) was associated with the lack of therapeutic responses to rituximab, a monoclonal antibody targeting CD20 expressed on B cells. These data indicate that the type I IFN signature is not a prognostic marker that universally predicts therapeutic responses of RA to potent antirheumatic drugs, but that it may differentiate patients who would preferentially benefit from a certain class of biologic agents.

Our data, taken together with the previous reports, suggest that both IL-6 and TNFα blocking therapies for RA are more likely to be efficacious when IFN activity is increased, and these data further support the notion that molecular and cellular mechanisms underlying the therapeutic effects of TCZ and TNF antagonists share at least a part of the same pathway in the pathophysiology of RA (3,32).

The molecular and cellular mechanisms by which the type I IFN signature plays a role in the responsiveness of RA to different biologic agents remain elusive. Although treatment with IFNβ is efficacious in some patients with relapsing remitting multiple sclerosis (MS), treatment with various forms of type I IFN (treatment that is also available for patients with hepatitis C) has been reported to cause or exacerbate other autoimmune diseases such as systemic lupus erythematosus (33), psoriasis (34), neuromyelitis optica (35), and RA (36).

A number of pathways have been postulated as underlying mechanisms for type I IFN–induced development of autoimmunity based on genetic or experimental data (23,37,38); however, only a few reports explain the difference between effects of IFNβ on MS, an archetypal autoimmune disease of the central nervous system, and the effects on other autoimmune diseases. Axtell et al reported that IFNβ promotes Th17 cell–mediated autoimmunity but attenuates Th1 cell–mediated autoimmunity and that the balance between Th17 and Th1 can determine the response of the autoimmune condition to IFNβ treatment (37,39). On the other hand, IL-6 signaling plays an important role in Th17 cell differentiation (40), and we recently identified down-regulated Th17 cell–related molecules in the CD4+ T cells of RA patients who received IL-6 blocking therapy (41). Since type I IFNs have been reported to enhance IL-6 signaling by providing docking sites for STAT-1 and STAT-3 on phosphorylated IFNα receptor 1 in close proximity to the gp130 chain of IL-6R (23,42), the increased expression of IFN response genes in PBMCs may reflect systemically increased type I IFN activities and subsequent IL-6–mediated Th17-driven inflammation, which can be readily antagonized by IL-6 blocking treatment.

MT1G encodes metallothionein-1G, a member of the metallothionein (MT) proteins, among which MT-1 and MT-2 are the most widely expressed isoforms in mammals. MT proteins are small, cysteine-rich proteins that bind to both essential and toxic metals and have been implicated in a range of roles including toxic metal detoxification and protection against oxidative stress (43–45). The MT-1 promoter contains a STAT binding site, and the gene expression of MT-1 is directly upregulated by IL-6 (45–47).

MT proteins have also been reported to be involved in immune and inflammatory responses, although the precise mechanism is not known (45,48–50). Given that the expression levels of *MT1G* were increased in RA patients who responded to IL-6–blocking treatment (Tables 2 and 3), *MT1G* expression in PBMCs may reflect the presence of increased IL-6 signaling, which is associated with systemic disease activity. Although the decrease in expression of *MT1G* after 3 months of effective TCZ treatment was not statistically significant (Figure 2), the decreases in the expression levels of *MT1B* and *MT2A*, the other MT genes identi-

fied in the training cohort, were statistically significant (data not shown). These data suggest that the gene expression of MT-1 and MT-2 may be synergistically up-regulated by IL-6/gp130/STAT-3 signaling in PBMCs, although other factors such as zinc concentration are also likely to be involved in the regulation of MT-1 and MT-2 gene expression (45).

Our study has several limitations. First, the sample size was not large enough to exclude Type I and Type II statistical errors, to perform multivariate analyses, or to stratify patients by background. In fact, the statistical significance of our data did not withstand correction for multiple testing. Also given that a previous study demonstrated that biomarkers that are identified as predictors of treatment responses in a single study are frequently unreproducible (51), our data need further confirmation. However, the number of patients who underwent genome-wide microarray analysis in our study is larger than that in previous studies of RA (9,11,29,52) and the 4 identified genes withstood statistical analyses using 2 independent cohorts and 2 different methods for gene expression. Moreover, 3 of the 4 genes (i.e., *IFI6*, *MX2*, and *OASL*) were IFN response genes and 3 genes encoding MT, other than *MT1G* (i.e., *MT1B*, *MT1L*, and *MT2A*), were also identified in the training cohort (Table 2). These data suggest that the final 4 genes were not incidentally identified by measurement errors but are likely to represent meaningful molecular pathways associated with the clinical consequences of IL-6 blockade treatment.

Second, therapeutic responses were determined by physician's global assessment, instead of established response criteria, such as EULAR response criteria. As mentioned in Patients and Methods, this method was chosen to avoid confounding the data with nonresponders who had nonspecific decreases in inflammatory responses as a result of IL-6 blockade (53). In fact, 2 nonresponders in our study were categorized as moderate responders when EULAR response criteria were applied, even though joint counts and the patients' global assessments of disease activity on the VAS did not improve at all (data not shown). We managed to distinguish nonresponders from true responders by reviewing comprehensive clinical information on an individual basis and by using the change in CDAI category as an objective reference; however, objective and standardized response criteria for TCZ need to be established. For this purpose, type I IFN signature could be a specific biomarker not only for predicting therapeutic responses, but also for monitoring therapeutic responses, given that all of the 3 identified IFN response genes were down-regulated only in responders in our study (Figure 2).

Third, although we intentionally focused on lymphocytes and monocytes that have been implicated in the pathogenesis of RA (3–5), gene expression analyses in PBMCs do not identify possibly informative genes that are preferentially expressed in granulocytes. Furthermore, isolating PBMCs is not always feasible in a typical clinical setting. Thus, whether whole blood cells are as informative as PBMCs in predicting therapeutic responses is a matter of great interest. To further improve the feasibility of applying this method to daily practice, soluble proteins in sera can be even more attractive biomarkers. Given the high discriminating capacity of the predictive models in our study, our data can be used to identify candidate serum biomarkers for use in predicting therapeutic responses to TCZ.

In conclusion, our study demonstrates that the expression levels of genes identified by genome-wide DNA microarray analyses can be predictive biomarkers for therapeutic responses to TCZ in RA patients. Our data provide valuable information for establishing strategies to optimize treatment with different classes of biologic agents. The results also indicate that type I interferon signaling and MT proteins are involved in the therapeutic responses of RA, providing insight into its molecular pathophysiology.

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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. Dr. Ikeda had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ikeda, Nakajima.

Acquisition of data. Sanayama, Ikeda, Saito, Kagami, Yamagata, Furuta, Kashiwakuma, Iwamoto, Umibe, Nawata, Matsumura, Sugiyama, Sueishi, Hiraguri, Nonaka, Ohara.

Analysis and interpretation of data. Sanayama, Ikeda, Nonaka, Ohara, Nakajima.

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AT-Rich–Interactive Domain–Containing Protein 5A Functions as a Negative Regulator of Retinoic Acid Receptor–Related Orphan Nuclear Receptor γt–Induced Th17 Cell Differentiation

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Objective. **The proinflammatory cytokines tumor necrosis factor α and interleukin-6 (IL-6) and the Th17 cell cytokine IL-17A are implicated in the pathogenesis of rheumatoid arthritis (RA), and the blockade of these cytokines by biologic agents provides clinical benefits for RA patients. We undertook this study to clarify the mechanisms underlying the efficacy of IL-6 blockade in RA and to find a novel target for treatment of RA.**

Methods. **We examined gene expression profiles of CD4+ T cells by DNA microarray analysis before and after treatment with an anti–IL-6 receptor antibody, tocilizumab (TCZ), in RA patients who exhibited good clinical responses to the treatment. Using murine CD4+ T cells, we then examined the roles of a newly identified molecule whose expression was significantly reduced in CD4+ T cells by TCZ therapy. We also examined the effect of the forced expression of the molecule on retinoic acid receptor–related orphan nuclear receptor γt (RORγt)–induced IL-17A production in CD4+ T cells and on RORγt-induced IL-17A promoter activation.**

Results. **We identified AT-rich–interactive domain–containing protein 5A (ARID-5A) as a new molecule down-regulated by IL-6 blockade in the form of TCZ therapy. IL-6 induced the expression of ARID-5A in CD4+ T cells during Th17 cell differentiation by a STAT-3–dependent mechanism, whereas IL-6–induced ARID-5A expression was not affected by the absence of RORγt, a lineage-specifying transcription factor of Th17 cells. Furthermore, ARID-5A physically associated with RORγt through its N-terminal region and inhibited RORγt-induced Th17 cell differentiation.**

Conclusion. **ARID-5A is a lineage-specific attenuator of Th17 cell differentiation and may be involved in the pathogenesis of RA.**

Rheumatoid arthritis (RA) is characterized by the destruction of cartilage and bone, with inflammation and cellular proliferation in the synovial joints. Accumulating evidence has shown that immune cells, including T cells, B cells, dendritic cells, and macrophages, play essential roles in the pathogenesis of RA (1). Proinflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), produced by these immune cells are involved not only in synovial inflammation, but also in extraarticular manifestations in RA (2,3). Clinical efficacy of biologic agents that block the effects of these proinflammatory cytokines has proved the roles of these cytokines in the pathogenesis of RA (4,5).

In addition to TNFα and IL-6, recent studies have demonstrated that Th17 cell–related cytokines such

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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

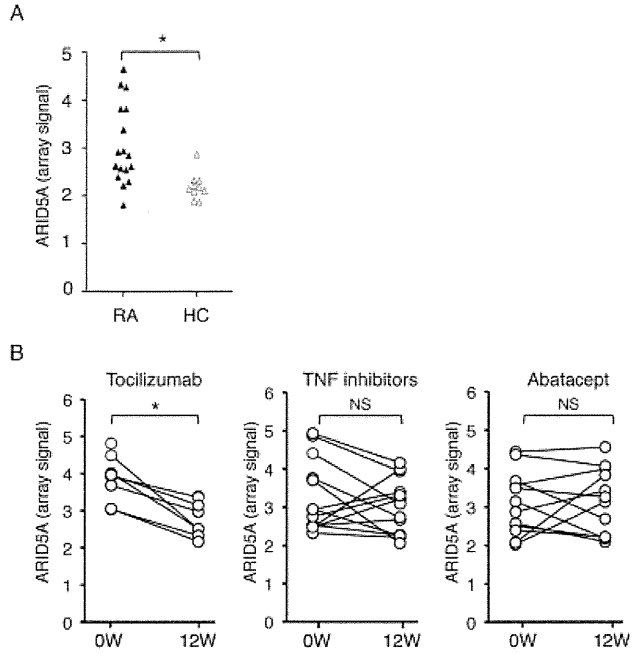

Figure 1. Expression of AT-rich–interactive domain–containing protein 5A (ARID-5A) in CD4+ T cells from rheumatoid arthritis (RA) patients is decreased by tocilizumab (TCZ) therapy. **A,** CD4+ T cells from untreated RA patients (n = 17) and those from healthy controls (HC) (n = 10) were subjected to 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

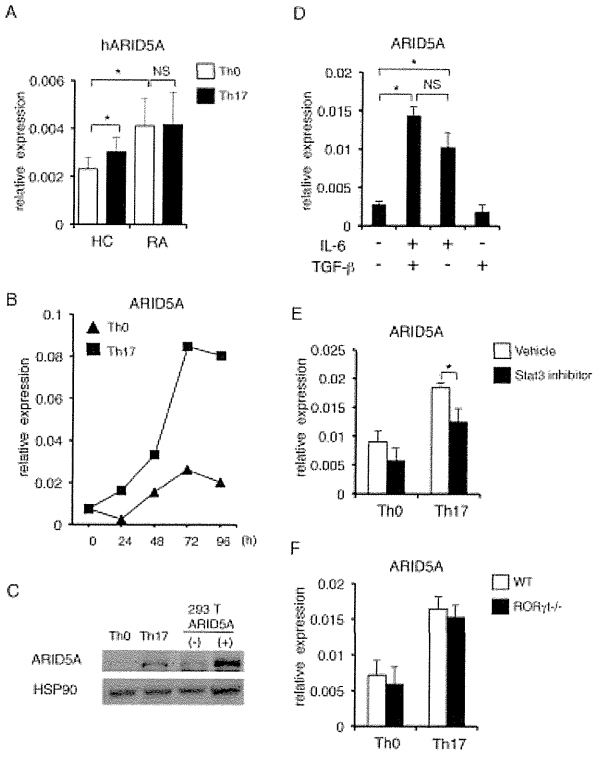

Figure 2. Interleukin-6 (IL-6)/STAT-3 signaling induces ARID-5A expression in human and murine Th17 cells. **A,** Memory CD4+ T cells from healthy controls (n = 6) or untreated RA patients (n = 6) were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions or Th17 cell–polarizing conditions, and 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

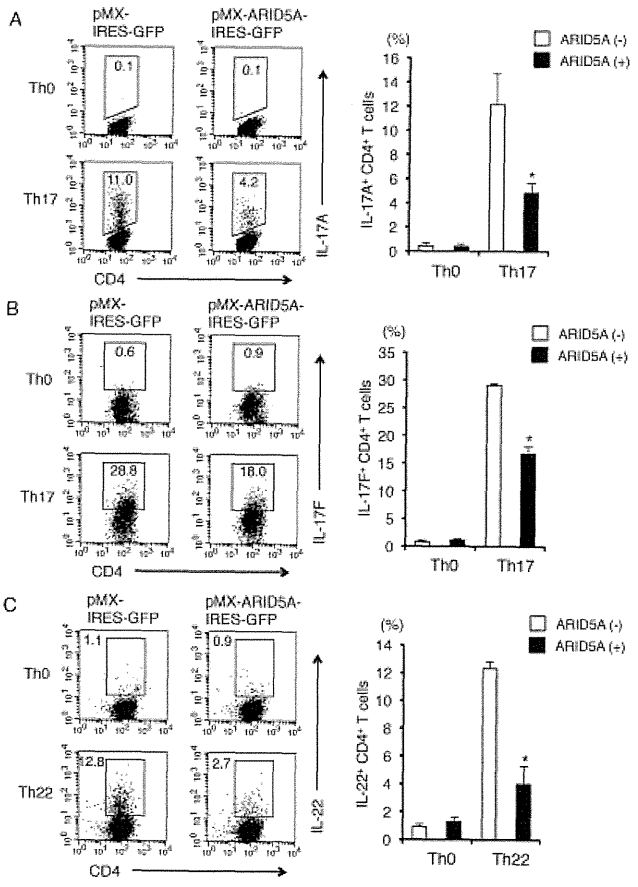

Figure 3. AT-rich–interactive domain–containing protein 5A (ARID-5A) inhibits Th17 cell differentiation. Murine naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions for 24 hours, and then cells were infected with a retrovirus of pMX-ARID5A-IRES-GFP (ARID-5A+) or pMX-IRES-GFP (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.

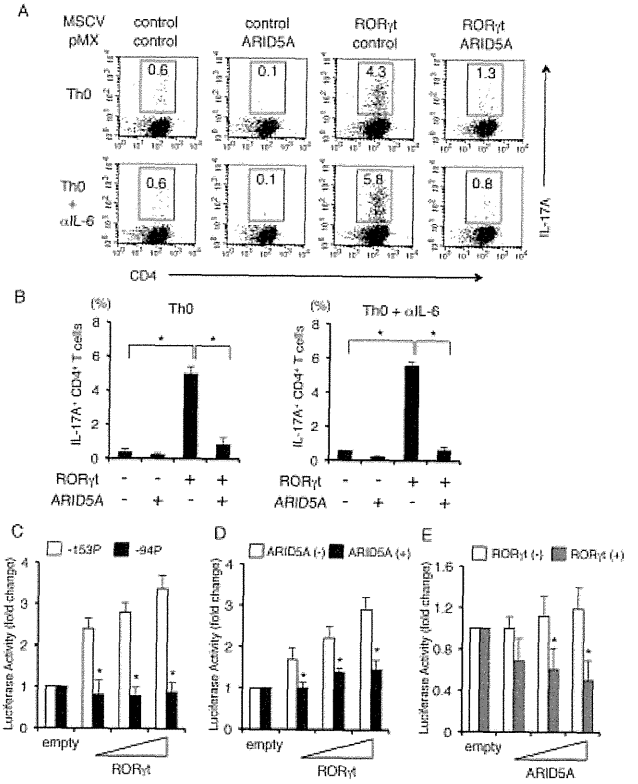

Figure 4. AT-rich–interactive domain–containing protein 5A (ARID-5A) inhibits retinoic acid receptor–related orphan nuclear receptor γt (RORγt)–induced Th17 cell differentiation. **A** and **B,** Murine naive CD4+ T cells were stimulated with anti-CD3/anti-CD28 under Th0 cell conditions for 24 hours 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.

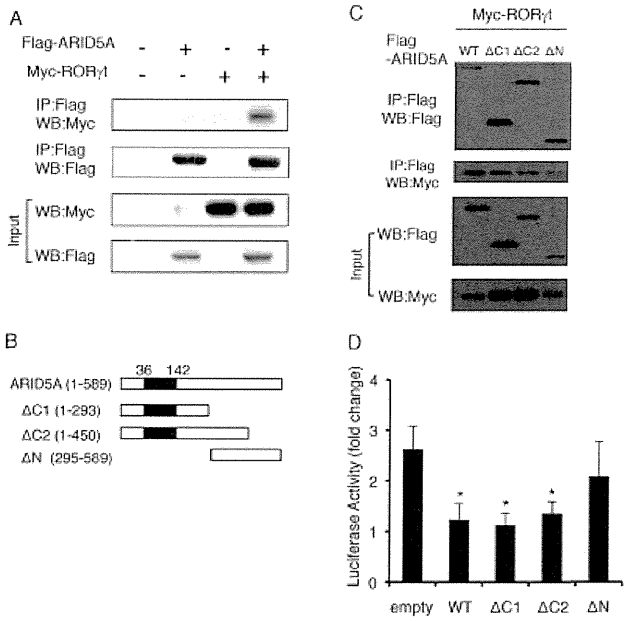

Figure 5. AT-rich–interactive domain–containing protein 5A (ARID-5A) physically associates with retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and inhibits its function. **A,** We cotransfected 293T cells with FLAG-tagged ARID-5A (pcDNA3-FLAG-ARID5A) and Myc-tagged RORγt (MSCV-myc-RORγt-IRES-Thy1.1). 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.

Study conception and design. Saito, Kagami, Suto, Nakajima.

Acquisition of data. Saito, Kagami, Sanayama, Ikeda, Kashiwakuma, Furuta, Nonaka, Ohara.

Analysis and interpretation of data. Saito, Kagami, Sanayama, Iwamoto, Nonaka, Ohara.

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