厚生労働科学研究費補助金

免疫アレルギー疾患等予防・治療研究事業

末梢血単核球の網羅的遺伝子発現解析による関節リウマチに対するトシリズマブの薬効予測と効果発現機序の解明に関する研究

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研究要旨

本研究では、末梢血単核球（PBMC）およびCD4陽性T細胞の網羅的遺伝子発現解析のデータをもとに、トシリズマブ（TCZ）の薬効予測モデルを構築し実用化すること、TCZの新規薬効評価マーカーを同定すること、TCZ効果発現の分子機構を解明することを目的とした。

TCZを新規に投与したRA患者を6ヵ月間経過観察し、治療効果判定を行った。TCZ治療前後のPBMCおよびCD4陽性T細胞における遺伝子発現の網羅的解析をDNAアレイを用いて行い、治療効果予測マーカーおよび治療効果判定マーカーの抽出を行った。候補マーカーについては半定量的PCRによる遺伝子発現の確認を行った。さらにTCZ有効例特異的に治療前後でCD4陽性T細胞において発現の変化する遺伝子を抽出し、そのCD4陽性T細胞分化における役割を解析した。

トレーニングコホートにおいて無効例と有効例でTCZ投与前のPBMCにおけるシグナル値に有意差を認め、かつ一定のシグナル強度を示す68プローブを同定した。それらの中で、重複して同定されたもの、同一ファミリー遺伝子、および免疫あるいは炎症反応に直接関連する19遺伝子23プローブに注目した。さらにそれらの中で、qPCRによる発現とDNAマイクロアレイシグナル値との相関が確認できた15遺伝子を予測候補遺伝子とした。バリデーションコホートにおいて、無効例と有効例の間でTCZ投与前のPBMCにおけるqPCRによる発現値に有意な差を認めたものは、15遺伝子中4遺伝子（*IFI6, MX2, OASL, MT1G*）であった。これらの遺伝子を用いたTCZの薬効予測モデルでは、ROC解析の曲線下面積が0.947、感度73.3%、特異度100%と良好な結果が得られた（Arthritis Rheumatol 2014;66:1421）。臨床情報ならびに関節超音波所見は、TCZの薬効予測には寄与しなかった。

ヒト末梢血およびマウス脾臓由来のナイーブCD4陽性T細胞から各種条件で分化誘導した細胞では、Th17細胞においてARID5AのmRNA発現が亢進していた。興味深いことに、マウス脾臓由来のCD4陽性T細胞にレトロウイルスベクターを用いてARID5Aを強制発現させると、Th17細胞分化が低下した。Treg細胞分化に対する影響はなかった。T細胞特異的STAT3欠損マウス由来のTh17細胞ではARID5AのmRNA発現が著明に低下していた。レポーター解析ではARID5Aの強制発現はRORγtによるIL-17発現を抑制し、またウェスタンブロットによりARID5AとRORγtの直接結合が示された（Arthritis Rheum 2014;66:1185）。

DNAマイクロアレイならびにqPCRによるPBMCの網羅的遺伝子発現解析により、RAにおけるTCZの薬効を高精度に予測する4遺伝子が同定された。RAの病態におけるI型IFNやメタロチオネインの関与が示唆された。またRA患者の末梢血CD4陽性T細胞において、TCZ投与により発現低下する遺伝子としてARID5Aが同定され、Th17細胞分化の新規抑制分子であることが示された。TCZ特異的薬効評価マーカーならびに新規治療ターゲット経路の候補と考えられた。

研究分担者氏名・所属研究機関名及び所属研究機関における職名

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A. 研究目的

本研究では、末梢血単核球（PBMC）およびCD4陽性T細胞の網羅的遺伝子発現解析のデータをもとに、トシリズマブ（TCZ）の薬効予測モデルを構築し実用化すること、TCZの新規薬効評価マーカーを同定すること、TCZ効果発現の分子機構を解明することを目的とした。

B. 方法

1）症例の組み入れ、評価項目、ならびに効果判定（池田、中島）

TCZを新規に投与したRA患者60症例（トレーニングコホート40症例、バリデーションコホート20症例）、またコントロールとしてメトトレキサート（MTX）、インフリキシマブ（IFX）、エタネルセプト（ETN）、アダリムマブ（ADA）ならびにアバタセプト（ABT）を新規に投与するRA患者それぞれ5症例ずつを24週間経過観察した。治療前、治療開始12週後、および治療開始24週後のDAS（Disease Activity Score）28、SDAI(Simplified Disease Activity Index)、CDAI(Clinical Disease Activity Index)、さらに関節超音波による38関節(DAS28関節+足関節+第2-5指MTP関節)のグレースケール（GS）およびパワードプラ（PD）の半定量スコア（OMERACTシステム）を記録した。治療24週での治療効果判定をCDAI変化量および医師総合評価により判定した。

2）末梢血単核球（PBMC）およびCD4陽性T細胞における遺伝子発現の網羅的解析（池田、中島、高取）

TCZ（n = 20)、TNF阻害薬（n = 15)、およびMTX（n = 5）を投与するRA患者の、治療前および治療開始12週後に全血20 mLをヘパリン採血した。速やかにPBMCを分離、さらにその3/4をCD4陽性T細胞へ純化し、それぞれの細胞における遺伝子発現をDNAアレイを用いて包括的に解析した。DNAアレイはかずさDNA研究所においてAgilent社製遺伝子発現用マイクロアレイHuman Whole Genomeを用いて行った。ラベル化方法はAgilent社製Quick Amp Labeling Kitを使用した。

3）TCZの治療効果を予測する臨床情報ならびに予測候補遺伝子の抽出（池田、中島）

トレーニングコホート(n = 20）の医師総合評価とCDAI変化による無効例と有効例において、有意差のある患者背景、およびTCZ治療開始前の臨床所見、血液検査所見、ならびに関節超音波所見を、単変量解析を用いて抽出した。同様にTCZ投与前のPBMCで発現に違いのある遺伝子を単変量解析で抽出した（t検定 $p<0.05$かつfold change>1.5）。

4）定量的PCRを用いたTCZの薬効予測モデルの確立（池田、廣瀬、中島）

3）により抽出された遺伝子の発現レベルを定量的リアルタイムPCRを用いて解析し、DNAマイクロアレイのシグナル値との相関を検証した。相関の高い遺伝子による薬効予測モデルを構築し、バリデーションコホート（n = 20）において薬効予測の精度を測定した。

5）血清マーカーを用いたTCZの薬効予測モデルの確立（池田）

4）により抽出された遺伝子より分泌蛋白、膜蛋白、ならびに関連血清蛋白を同定し、トレーニングコホートの有効群と無効群の血清における蛋白発現の差をELISAを用いて確認した。

6）TCZの効果発現機序の解明（池田、中島、高取）

TCZ著効症例の末梢血CD4陽性T細胞において、TCZ投与後に発現の低下しているAT-rich interactive domain-containing protein 5a（ARID5A)に注目した。ヒト末梢血およびマウス脾臓由来のナイーブCD4陽性T細胞から分化誘導したTh1細胞、Th2細胞、Th17細胞、Tfh細胞、Treg細胞におけるARID5Aの発現をリアルタイムPCR法で解析した。マウス脾臓由来のCD4陽性T細胞にレトロウイルスベクターを用いてARID5AをGFPと共に強制発現させ、各ヘルパーT細胞の分化誘導条件で培養した際のヘルパーT細胞分化への影響を細胞内サイトカイン染色法により解析した。またT細胞特異的STAT3/RORγt欠損マウスの脾臓由来のナイーブCD4陽性T細胞を各種条件で分化誘導した際のARID5Aの発現を解析した。

7）新規薬効評価マーカーの同定（高取、池田）

全症例において、CDAI/超音波総PDスコアと同時に採取したPBMC/CD4陽性T細胞での発現が相関する遺伝子を抽出した。また有効群と無効群の間で、投与12週での発現の変化の差が大きい遺伝子を抽出した。これらを発現量、あるいは分泌蛋白、膜蛋白で絞り込み、定量的PCRあるいはELISAで発現を確認した。治療12週でのSRM（standardized response mean)、あるいは52週での総シャープスコアとの相関を解析することにより、新規薬効評価マーカーとしての有用性を検討した。

C. 結果

トレーニングコホートにおいて無効例（n=8）と有効例（n=29）でTCZ投与前のPBMCにおけるシグナル値に有意差を認め（図1)、かつ一定のシグナル強度を示す68プローブを同定した。それらの中で、重複して同定されたもの、同一ファミリー遺伝子、および免疫あるいは炎症反応に直接関連する19遺伝子23プローブに注目した。さらにそれらの中で、qPCRによる発現とDNAマイクロアレイシグナル値との相関が確認できた15遺伝子を予測候補遺伝子とした。バリデーションコホートにおいて、無効例（n=5）と有効例（n=15）の間でTCZ投与前のPBMCにおけるqPCRによる発現値に有意な差を認めたものは、15遺伝子中4遺伝子（*IFI6, MX2, OASL, MT1G*）であった（表1)。これらの遺伝子を用いたTCZの薬効予測モデルでは、Receiver operating characteristic（ROC)解析の曲線下面積が0.947、感度73.3%、特異度100%と良好な結果が得られた（Arthritis Rheumatol 2014;66:1421）。臨床情報ならびに関節超音波所見は、TCZの薬効予測には寄与しなかった。

ヒト末梢血およびマウス脾臓由来のナイーブCD4陽性T細胞から各種条件で分化誘導した細胞では、Th17細胞においてARID5AのmRNA発現が亢進していた。興味深いことに、マウス脾臓由来のCD4陽性T細胞にレトロウイルスベクターを用いてARID5Aを強制発現させると、Th17細胞分化が低下した（図2）。Treg細胞分化に対する影響はなかった。T細胞特異的STAT3欠損マウス由来のTh17細胞ではARID5AのmRNA発現が著明に低下していた。レポーター解析ではARID5Aの強制発現はRORγtによるIL-17発現を抑制し（図3)、またウェスタンブロットによりARID5AとRORγtの直接結合が示された（Arthritis Rheum 2014;66:1185)。

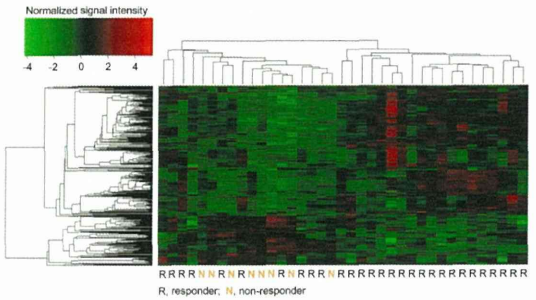

図 1. 無効例と有効例の間でシグナル値が有意に異なったプローブのクラスター解析

Gene	Expression relative to GAPDH, mean ± SD		P
	Nonresponders	Responders	
CCL3L3	0.22908 ± 0.11719	0.71191 ± 1.14197	0.128
CCL4	0.47432 ± 0.52809	0.32468 ± 0.16105	0.565
CD83	0.09453 ± 0.06540	0.12588 ± 0.20742	0.623
CXCR4	2.02442 ± 0.52763	2.60166 ± 1.34114	0.186
FOSL2	0.12600 ± 0.05244	0.15703 ± 0.13811	0.477
HP	0.01959 ± 0.01192	0.03599 ± 0.03659	0.148
IFI6	0.01166 ± 0.00514	0.01517 ± 0.01106	0.038
LY6E	0.21193 ± 0.09510	0.41288 ± 0.45754	0.128
MT1G	0.00039 ± 0.00030	0.00164 ± 0.00128	0.003
MT2A	0.26977 ± 0.10763	0.35474 ± 0.24362	0.299
MX2	0.07054 ± 0.02718	0.13847 ± 0.08220	0.012
OASL	0.03208 ± 0.00883	0.07313 ± 0.06817	0.038
RABGEF1	0.03439 ± 0.01607	0.05279 ± 0.03053	0.107
THBS1	0.12593 ± 0.10264	0.24968 ± 0.26263	0.149
WARS	0.42023 ± 0.15457	0.51446 ± 0.28856	0.371

表1. バリデーションコホートのTCZ投与前のPBMCにおける15遺伝子の発現

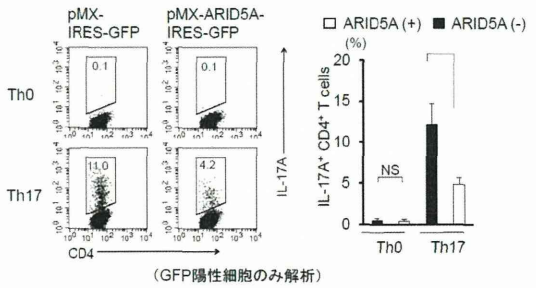

図2. マウスCD4陽性T細胞におけるARID5Aの強制発現によるTh17細胞分化の低下

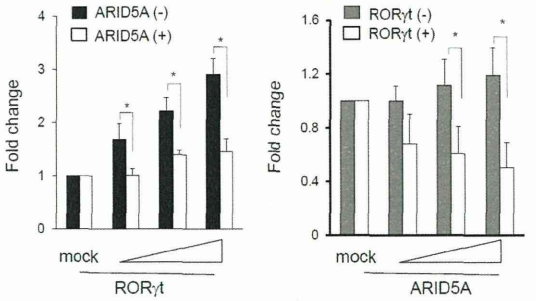

図3. ARID5AおよびRORγtの強制発現によるIL-17プロモーター活性の変化

D. 考察

TCZ 薬効予測遺伝子として抽出された4遺伝子中、*IFI6*, *MX2*, *OASL* はいずれも Type I interferon (IFN) response gene (IRG)であり、その他候補となった 19 遺伝子のうち *LY6E* も IRG であった。IRG の末梢血における高発現は、TNF 阻害薬においても良好な薬効予測因子であることが報告されているが、B 細胞標的薬であるリツキシマブでは逆に治療不応性の予測因子であることが示されており、IRG 発現は薬剤選択に有用なバイオマーカーであることが示唆される。I 型 IFN は Th17 分化により自己免疫を促進することが示されており、そのような病態においては IL-6 シグナル阻害による Th17 分化抑制が RA の病態改善に有効である可能性が示唆された。また *MT1G* はメタロチオネインファミリー遺伝子の1つであるが、メタロチオネインは IL-6 により直接発現誘導される。メタロチオネイン遺伝子発現が IL-6 依存性炎症を反映している可能性が示唆された。現在 4 遺伝子によるカスタムアレイを作成中であり、関連血清マーカーとともに大規模での TCZ の薬効予測精度を検証予定である。

ARID5A は Th17 分化に伴って STAT3 依存性に発現し、Th17 細胞分化を抑制する負のフィードバック機構として働いていること、その分子機構として RORγt との直接結合による阻害が示唆された。近年 ARID5A の IL-6 mRNA の安定性を介した自己免疫への関与が報告されているが、その関節炎モデルにおける役割を現在検討中である。

複数の IRGs、メタロチオネイン I/II ファミリー遺伝子、さらに ARID5A は、TCZ 有効群において疾患活動性の低下に伴いその発現が低下した。TCZ 投与下における特異的な疾患活動性/薬効評価マーカーとしての有用性が示唆され、他の抗リウマチ薬投与患者を含め検証中である。

E. 結論

DNA マイクロアレイならびに qPCR による PBMC の網羅的遺伝子発現解析により、RA における TCZ の薬効を高精度に予測する4遺伝子が同定された。RA の病態における I 型 IFN やメタロチオネインの関与が示唆された。また RA 患者の末梢血 CD4 陽性 T 細胞において、TCZ 投与により発現低下する遺伝子として ARID5A が同定され、Th17 細胞分化の新規抑制分子であることが示された。TCZ 特異的薬効評価マーカーならびに新規治療ターゲット経路の候補と考えられた。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

「関節リウマチに対する抗 IL-6 受容体抗体療法の有効性の予測方法」特願 2011-156921

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

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Prediction of Therapeutic Responses to Tocilizumab in Patients With Rheumatoid Arthritis

Biomarkers Identified by Analysis of Gene Expression in Peripheral Blood Mononuclear Cells Using Genome-Wide DNA Microarray

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Objective. **The aim of this prospective multicenter study was to identify biomarkers that can be used to predict therapeutic responses to tocilizumab in patients with rheumatoid arthritis (RA).**

Methods. **We recruited patients with RA who were treated with tocilizumab for the first time, and determined therapeutic responses at 6 months. In the training cohort (n = 40), gene expression in peripheral blood mononuclear cells (PBMCs) at baseline was analyzed using genome-wide DNA microarray, with 41,000 probes derived from 19,416 genes. In the validation cohort (n = 20), expression levels of the candidate genes in PBMCs at baseline were determined using real-time quantitative polymerase chain reaction (qPCR) analysis.**

Results. **We identified 68 DNA microarray probes that showed significant differences in signal intensity between nonresponders and responders in the training cohort. Nineteen putative genes were selected, and a significant correlation between the DNA microarray signal intensity and the qPCR relative expression was confirmed in 15 genes. In the validation cohort, a significant difference in relative expression between nonresponders and responders was reproduced for 3 type I interferon response genes (*IFI6, MX2,* and *OASL*) and *MT1G*. Receiver operating characteristic curve analysis of models incorporating these genes showed that the maximum area under the curve was 0.947 in predicting a moderate or good response to tocilizumab in the validation cohort.**

Conclusion. **Using genome-wide DNA microarray analyses, we identified candidate biomarkers that can be used to predict therapeutic responses to tocilizumab in patients with RA. These findings suggest that type I interferon signaling and metallothioneins are involved in the pathophysiology of RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints, which

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cause disability and premature mortality (1,2). Accumulating evidence supports the notion that immune cells, including T cells, B cells, dendritic cells, and the macrophages and proinflammatory cytokines produced by these cells (such as tumor necrosis factor α [TNFα] and interleukin-6 [IL-6]), play essential roles in the pathogenesis of RA (3–5). In fact, treatment with biologic antirheumatic drugs, which specifically inhibits these cytokines or cellular interactions, has substantially improved clinical, structural, and functional outcomes of RA (2,4,6). However, treatment with a biologic agent is not universally efficacious in all patients, reflecting the heterogeneous molecular and cellular mechanisms underlying the pathophysiology of RA (4,5).

Considering the cost and the risk of infection, biologic agents should be prescribed only to patients in whom therapeutic responses to the drug are likely to be achieved. However, studies that assessed the predictive values of clinical and laboratory information for therapeutic responses to TNF antagonists have yielded neither consistent results nor clinically applicable strategies. Although some genetic factors have been identified as potentially predictive markers of therapeutic responses to TNF antagonists, a meta-analysis and data from a large registry showed no association between therapeutic response to TNF antagonists and TNF-308 polymorphisms (7) or shared epitope motifs (8), the genetic markers that have been considered the most promising candidates. These data suggest that clinical information and genetic markers may not be accurate predictors of responses to treatment with biologic agents.

DNA microarray analysis enables assessment of expression of messenger RNA (mRNA) for multiple genes in the target cells or tissues. Previous studies showed that mRNA expression analyses of a set of genes using microarray techniques predicted clinical responses to infliximab (9–12) or rituximab (13) in RA patients. These data indicate that DNA microarray is a powerful tool that can be used to identify genes that may be biomarkers for the prediction of clinical responses to certain antirheumatic treatments in RA patients.

Tocilizumab (TCZ) is a humanized anti–IL-6 receptor (anti–IL-6R) monoclonal antibody that inhibits IL-6 signaling by blocking the binding of IL-6 to IL-6R. Although the overall rate of response to TCZ in RA patients is high (14–16), the improvement of synovitis in these patients is frequently slow to become clinically apparent as compared with improvement seen in patients treated with TNF antagonists. Therefore, it can take months to determine whether TCZ treatment has lacked effectiveness, thereby possibly exposing patients to unnecessary risks, including damage progression and side effects, at a disproportionate cost. Thus, predicting therapeutic responses can be particularly beneficial for TCZ, but such methods of prediction, with sufficient accuracy, have not been established yet. This prospective multicenter study aimed to identify biomarkers that can be used to predict therapeutic responses to TCZ in patients with RA. We used genome-wide DNA microarray to analyze comprehensive gene expression in peripheral blood mononuclear cells (PBMCs).

PATIENTS AND METHODS

Patients and healthy subjects. Patients who fulfilled the American College of Rheumatology (ACR) 1987 revised criteria for the classification of RA (17) and who received treatment with TCZ for the first time at a participating hospital (Chiba University Hospital, Asahi General Hospital, Matsudo City Hospital, Chibaken Saiseikai Narashino Hospital, National Hospital Organization Chiba-East Hospital, National Hospital Organization Shimoshizu Hospital, or Narita Red Cross Hospital) were consecutively recruited. Forty patients were recruited for the training cohort for identification of candidate genes, and another 20 patients were recruited for the validation cohort for confirming predictive values of these genes. Patients received routine clinical care and underwent clinical and laboratory assessment at baseline and at 3 and 6 months after initiation of TCZ treatment. Healthy subjects who did not have any arthritis symptoms were also recruited as controls. The study design was approved by the Ethics Committee of Chiba University, and written informed consent was obtained in accordance with the Declaration of Helsinki.

Clinical and laboratory assessment. Clinical and laboratory assessment included 28-joint counts for swelling and tenderness, patient's global assessment and physician's global assessment of disease activity on a visual analog scale (VAS), Health Assessment Questionnaire Disability Index (18), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) level. Rheumatoid factor and anti–cyclic citrullinated protein antibody levels were investigated at baseline only.

Response to therapy with TCZ. Because IL-6 blockade with TCZ substantially decreases markers of acute inflammation, such as ESR and serum CRP levels, regardless of therapeutic response (19,20), response criteria that include these markers, such as ACR or European League Against Rheumatism (EULAR) response criteria, were not used in this study. Instead, clinical responses to TCZ treatment were determined primarily by physician's global assessment (good/moderate/no response) at 6 months. This assessment was determined by consensus among the physicians (Yoshie Sanayama, KI, SK, SF, DK, TU, YN, RM, TS, MS, or MH) and an independent rheumatologist (Yoshie Sanayama or KI) through a review of comprehensive clinical information. The change in Clinical Disease Activity Index (CDAI) category (high = >22, moderate = >10–22, low = >2.8–10, or remission = ≤2.8) (21) at 6 months was also used to supplement the physician's global assessment.

Table 1. Baseline characteristics of the patients in the training and validation cohorts*

Baseline variable	Training cohort			Validation cohort		
	Total (n = 40)†	Nonresponder (n = 8)	Responder (n = 29)	Total (n = 20)	Nonresponder (n = 5)	Responder (n = 15)
Age, mean ± SD years	58.4 ± 15.2	54.3 ± 10.2	59.5 ± 16.8	60.9 ± 9.9	61.6 ± 11.1	60.7 ± 9.8
Female, no. (%)	31 (78)	5 (63)	23 (79)	16 (80)	4 (80)	12 (80)
Disease duration, median (IQR) months	57.5 (18.3–173.8)	92 (25.3–188.8)	58 (13–171)	44.5 (19–120.8)	46 (43–359)	39 (17.5–94)
Rheumatoid factor positive, no. (%)	34 (85)	7 (88)	25 (86)	18 (90)	4 (80)	14 (93)
ACPA positive, no. (%)	34 (85)	8 (100)	25 (86)	15 (75)	4 (80)	11 (73)
Antinuclear antibody positive, no. (%)	24 (60)	4 (50)	19 (66)	8 (40)	2 (40)	6 (40)
Anti-SSA antibody positive, no. (%)	6 (15)	0 (0)	6 (21)	5 (25)	1 (20)	4 (27)
Extraarticular manifestation present, no (%)	11 (28)	4 (50)	6 (21)	1 (5)	0 (0)	1 (7)
Smoking history, no. (%)						
Never smoked	28 (70)	6 (75)	20 (69)	16 (80)	5 (100)	11 (73)
Ex-smoker	10 (25)	2 (25)	7 (24)	1 (5)	0 (0)	1 (7)
Current smoker	2 (5)	0 (0)	2 (7)	3 (15)	0 (0)	3 (20)
Tender joint count, median (IQR)	5 (1–7.8)	5 (0.8–10.5)	5 (1–7)	4 (2–8.3)	4 (2–8)	4 (1.5–7.5)
Swollen joint count, median (IQR)	8.5 (4–11)	7 (3.5–9.3)	9 (5–12)	8 (2–9.5)	2 (1–3)	8 (2.5–10)
CDAI, median (IQR)	24.2 (20.3–31.23)	25.25 (19.8–28.8)	24.9 (20.5–31.9)	21.4 (14.95–25)	19.5 (13–21.8)	22.6 (15.85–25)
ESR, median (IQR) mm/hour	44 (30.3–68.8)	47.5 (28.5–79.8)	44 (32–67)	52 (36–67)	45 (37–61)	55.5 (37.8–70.8)
CRP, median (IQR) mg/dl	2.355 (0.75–4.1)	2.375 (1.528–5.11)	2.41 (0.79–4.1)	1.025 (0.415–2.585)	0.99 (0.9–1.05)	1.52 (0.38–3.275)
DAS28 score based on ESR, median (IQR)	5.35 (4.475–6.033)	5.365 (4.145–6.388)	5.34 (4.595–5.975)	5.09 (4.41–5.47)	4.73 (4.44–5.33)	5.21 (4.46–5.485)
MMP-3 level, median (IQR) ng/ml	199.5 (121.5–323.75)	233.5 (121.3–358.0)	196 (124–320)	224 (125–328.5)	138 (133–245.8)	239 (134–364.5)
Dosage of MTX, median (IQR) mg/week	8 (0–10.5)	6 (4.5–7.6)	8 (0–12.5)	6.8 (3–13)	10 (6–12)	6 (0–14)
Dosage of prednisolone, median (IQR) mg/day	3.9 (0–5)	5.5 (2.8–6.8)	3 (0–5)	1 (0–5)	0 (0–2.5)	1 (0–5)
Treatment with TNF antagonists, no. (%)						
Never	15 (38)	3 (38)	12 (41)	7 (35)	2 (40)	5 (33)
Previous	3 (8)	1 (13)	2 (7)	4 (20)	1 (20)	3 (20)
Current	22 (55)	4 (50)	15 (52)	9 (45)	2 (40)	7 (47)

* There were no statistically significant differences between responders and nonresponders in either the training cohort or the validation cohort. IQR = interquartile range; ACPA = anti–citrullinated protein antibody; CDAI = Clinical Disease Activity Index; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints; MMP-3 = matrix metalloproteinase 3; MTX = methotrexate; TNF = tumor necrosis factor.

† Three patients were not classified as responder or nonresponder because tocilizumab treatment was discontinued before they had received 3 months of treatment.

DNA microarray analysis. At baseline, PBMCs from patients in the training and validation cohorts, as well as from healthy controls, were isolated using Ficoll-Paque Premium 1.073 (GE Healthcare). PBMCs were also isolated from patients in the training cohort at 3 months (which is earlier than the time at which a therapeutic response can be determined) to assess the more direct effects of TCZ on gene expression. Total cellular RNA was extracted from PBMCs using Isogen solution (Nippon Gene). For patients in the training cohort and for healthy controls, DNA microarray analysis was performed using a Quick Amp labeling kit and a Whole Human Genome DNA Microarray 4×44K according to the protocol of the manufacturer (Agilent). Microarray data were analyzed using GeneSpring GX11.5.1 software (Agilent). Signal intensity was normalized by adjusting data to a 75th percentile baseline.

Real-time quantitative polymerase chain reaction (qPCR) analysis. Real-time qPCR analysis was performed in both the training and validation cohorts at baseline. The genes and the corresponding primers used are listed in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38400/abstract. Reverse transcription of extracted RNA was performed using an iScript cDNA Synthesis kit (Bio-Rad). Expression levels were measured with an ABI Prism 7300 instrument according to the standard protocol recommended

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

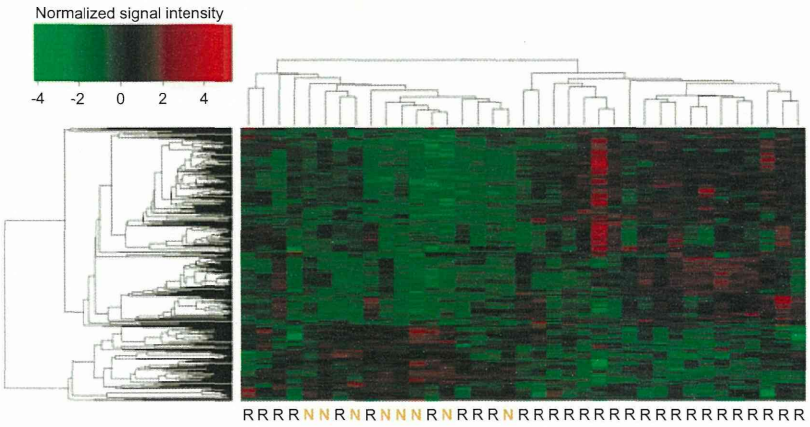

Figure 1. Hierarchical clustering of signal intensity patterns for individual patients and DNA microarray probes, identified by comparing those who responded to tocilizumab treatment with those who did not respond to tocilizumab treatment in the training cohort. The heatmap shows the normalized signal 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		P	Expression relative to ubiquitin C, mean ± SD		P
	Nonresponders	Responders		Nonresponders	Responders	
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

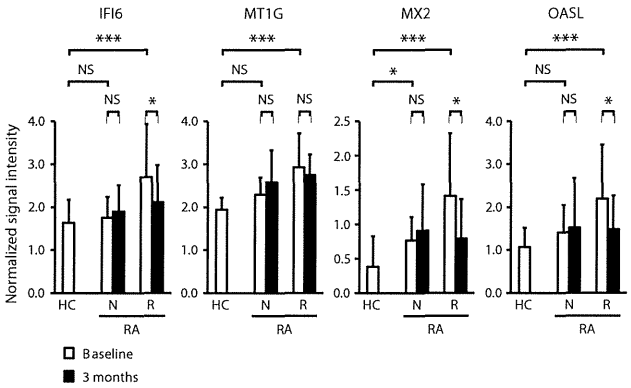

Figure 2. Comparisons of DNA microarray signal intensities between healthy controls (HCs), rheumatoid arthritis (RA) patients who were classified as nonresponders (N), and RA patients who were classified as responders (R), and comparisons of DNA microarray signal intensities within each group between 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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