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Crucial Role of the Interleukin-6/Interleukin-17 Cytokine Axis in the Induction of Arthritis by Glucose-6-Phosphate Isomerase

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Objective. **To clarify the glucose-6-phosphate isomerase (GPI)–specific CD4+ T cell lineage involved in GPI-induced arthritis and to investigate their pathologic and regulatory roles in the induction of the disease.**

Methods. **DBA/1 mice were immunized with GPI to induce arthritis. CD4+ T cells and antigen-presenting cells were cocultured with GPI, and cytokines in the supernatant were analyzed by enzyme-linked immunosorbent assay. Anti–interferon-γ (anti-IFNγ) monoclonal antibody (mAb), anti–interleukin-17 (anti–IL-17) mAb, or the murine IL-6 receptor (IL-6R) mAb MR16-1 was injected at different time points, and arthritis development was monitored visually. After mAb MR16-1 was injected, percentages of Th1, Th2, Th17, and Treg cells were analyzed by flow cytometry and CD4+ T cell proliferation was analyzed using carboxyfluorescein diacetate succinimidyl ester.**

Results. **GPI-specific CD4+ T cells were differentiated to Th1 and Th17 cells, but not Th2 cells. Administration of anti–IL-17 mAb on day 7 significantly ameliorated arthritis ($P < 0.01$), whereas administration of anti-IFNγ mAb exacerbated arthritis. Neither anti–IL-17 mAb nor anti-IFNγ mAb administration on day 14 ameliorated arthritis. MR16-1 administration on day 0 or day 3 protected against arthritis induction, and MR16-1 administration on day 8 significantly ameliorated existing arthritis ($P < 0.05$). After administration of MR16-1, there was marked suppression of Th17 differentiation, without an increase in Th1, Th2, or Treg cells, and CD4+ T cell proliferation was also suppressed.**

Conclusion. **IL-6 and Th17 play an essential role in GPI-induced arthritis. Since it has previously been shown that treatment with a humanized anti–IL-6R mAb has excellent effects in patients with rheumatoid arthritis (RA), we propose that the IL-6/IL-17 axis might also be involved in the generation of RA, especially in the early effector phase.**

Rheumatoid arthritis (RA) is characterized by symmetric polyarthritis and joint destruction. Although the etiology of RA is considered to be an autoimmune reactivity to antigens that are specifically expressed in joints, this remains a controversial hypothesis. It has been reported that autoimmune reactivity to a ubiquitous cytoplasmic enzyme, glucose-6-phosphate isomerase (GPI), provokes joint-specific inflammation in K/BxN mice (1,2). This finding highlights the potential role of systemic autoreactivity to certain ubiquitous autoantigens in the pathogenesis of RA.

More recently, it was reported that arthritis can also be induced in DBA/1 mice by immunization with GPI (3). GPI-induced arthritis is different from collagen-induced arthritis (CIA) with regard to the priority of T cells and B cells. In CIA, treatment with anti-CD4 monoclonal antibodies (mAb) is ineffective after the mice have produced antibodies to type II collagen (4,5), and CD4-deficient mice can develop CIA

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at the same incidence and severity as untreated mice (6). Adoptive transfer of IgG antibodies purified from mice with CIA can induce arthritis even in strains that are not susceptible to CIA induction by conventional immunization. In GPI-induced arthritis, administration of anti-CD4 mAb rapidly ameliorates arthritis after its onset, despite the absence of changes in the anti-GPI antibody titers. Fcγ receptor–deficient mice are resistant to GPI-induced arthritis, and adoptive transfer of purified IgG antibodies alone is not able to induce arthritis in these mice (3). These findings indicate that although autoantibodies are necessary for GPI-induced arthritis, CD4+ T cells are indispensable even after antibody production.

The present study was designed to further characterize the importance of CD4+ T cells in GPI-induced arthritis. Specifically, we investigated the CD4+ T cell lineage involved in GPI-induced arthritis and the regulatory mechanisms of pathogenic T cells. The results demonstrated that GPI-specific CD4+ T cells shifted to Th1 and Th17 cells and that Th17 played a crucial role in the development of GPI-induced arthritis. We also found that blockade of interleukin-6 receptor (IL-6R) significantly suppressed the arthritis and inhibited Th17 differentiation. The main message of this study is that the IL-6/IL-17 axis may be essential for the development of T cell–dependent autoimmune arthritis.

MATERIALS AND METHODS

Mice. Male DBA/1 mice were purchased from Charles River Laboratories (Yokohama, Japan). All mice were maintained under specific pathogen–free conditions, and all experiments were conducted in accordance with the institutional ethics guidelines.

GPI-induced arthritis. Recombinant human GPI was prepared as described previously (7). Briefly, human GPI complementary DNA was inserted into plasmid pGEX-4T3 (Pharmacia, Uppsala, Sweden) for expression of glutathione S-transferase–tagged proteins. The *Escherichia coli*–harboring pGEX-hGPI plasmid was allowed to proliferate at 37°C before the addition of 0.1 m*M* IPTG to the medium, which was followed by a further culture overnight at 30°C. The bacteria were lysed with a sonicator, and the supernatant was purified with a glutathione–Sepharose column (Pharmacia). The purity was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Mice were immunized intradermally with 300 μg of recombinant human GPI in Freund's complete adjuvant (Difco, Detroit, MI). Recombinant human GPI and Freund's complete adjuvant were emulsified at a 1:1 ratio (volume/volume). For induction of arthritis, 150 μl of the emulsion was injected intradermally into the base of the tail. For intracellular staining and cell proliferation assay, 50 μl was injected into each footpad of the hind paw. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0–3, where 0 = no evidence of inflammation, 1 = subtle inflammation or localized edema, 2 = easily identified swelling that was localized to either the dorsal or ventral surface of the paw, and 3 = swelling of all aspects of the paw.

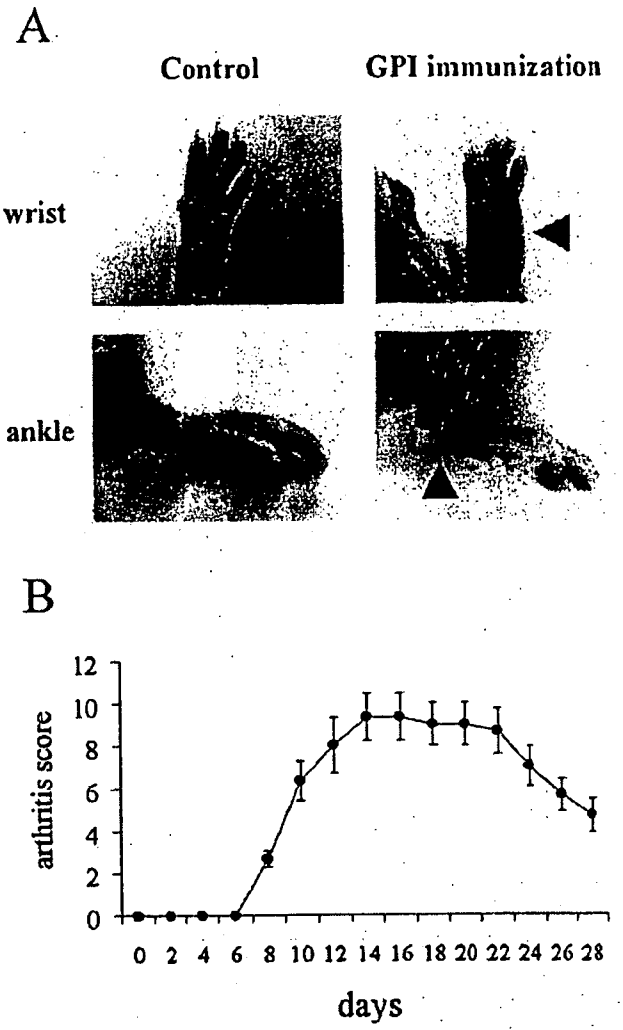

Figure 1. Induction of severe polyarthritis by immunization with recombinant human glucose-6-phosphate isomerase (GPI). DBA/1 mice were immunized with 300 μg of recombinant human GPI, and the development of arthritis was monitored visually and scored on a scale of 0–3 (see Materials and Methods for details). Arthritis was clinically apparent beginning on days 7–8, peaked in severity on day 14, and then gradually subsided. A, Severe swelling of the wrist and ankle joints (arrowheads) in mice immunized with GPI as compared with control mice. B, Mean ± SEM arthritis scores on days 0–28 in 10 mice from a representative experiment.

Analysis of cytokine profiles. Mice were killed on day 7 or day 14. Spleens were harvested and hemolyzed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$, and 0.004% disodium EDTA in phosphate buffered saline (PBS). Single-cell suspensions were prepared in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum

(FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 50 μ*M* 2-mercaptoethanol. CD4+ T cells were isolated by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity (>97%) was confirmed by flow cytometry. Splenic feeder cells treated with 50 μg/ml of mitomycin C were used as antigen-presenting cells (APCs). Purified CD4+ T cells and APCs were cocultured with 5 μg/ml of GPI at a ratio of 5:1 for 24 hours at 37°C in an atmosphere containing 5% CO_2. The supernatants were assayed for interferon-γ (IFNγ), IL-4, and IL-17 by enzyme-linked immunosorbent assay (ELISA) using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Treatment of arthritis with antibodies. To neutralize IL-17 and IFNγ, mice were injected intraperitoneally with 100 μg of neutralizing antibody or isotype control on day 7 or day 14. Anti–IL-17 mAb MAB421 (IgG2a) and anti-IFNγ mAb MAB485 (IgG2a) were purchased from R&D Systems. IgG2a isotype control was purchased from eBioscience (San Diego, CA). For IL-6 neutralization, mice were injected intraperitoneally with 2 mg or 4 mg of MR16-1 (an IgG1-specific mAb against murine IL-6R) or control IgG (purified from the serum of nonimmunized rats) on day 0, 3, 8, or 14. MR16-1 was a gift from Chugai Pharmaceutical (Tokyo, Japan), and control IgG was purchased from Jackson ImmunoResearch (West Grove, PA).

Anti-GPI antibody analysis. Sera were obtained on day 28 or day 35 and diluted 1:500 in blocking solution (25% Block-Ace [Dainippon Sumitomo Pharma, Osaka, Japan] in PBS) for analysis of antibody. Then, 96-well plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 5 μg/ml of recombinant human GPI for 12 hours at 4°C. After washing twice with washing buffer (0.05% Tween 20 in PBS), the blocking solution was applied for 2 hours at room temperature to block nonspecific binding. After 2 washes, 150 μl of diluted sera was added, and the plates were incubated for 2 hours at room temperature. After 3 washes, alkaline phosphatase (AP)–conjugated anti-mouse IgG was added at a final dilution of 1:5,000 for 1 hour at room temperature. After 3 washes, color was developed with substrate solution, consisting of 1 tablet of AP tablet (Sigma-Aldrich) per 5 ml of AP reaction solution (9.6% diethanolamine and 0.25 m*M* $MgCl_2$, pH 9.8). Plates were incubated for 20 minutes at room temperature, and the optical density was read at 405 nm using a microplate reader.

Intracellular cytokine staining and flow cytometric analysis. Mice were killed on day 7. Popliteal lymph nodes were harvested, and single-cell suspensions were prepared as described above. Cells (1×10^6/ml) were stimulated with 100 μg/ml of recombinant human GPI in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) for 24 hours. GoldiStop (BD PharMingen, San Diego, CA) was added during the last 2 hours of each culture. Cells were stained extracellularly, fixed, and permeabilized with Cytofix/Cytoperm solution (BD PharMingen), then the cells were stained intracellularly. A mouse regulatory T cell staining kit (eBioscience) was used to stain Treg cells according to the protocol supplied by the manufacturer. Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). AQ: 6

Cell proliferation assay. Mice were killed on day 10. Popliteal lymph nodes were harvested, and single-cell suspensions were prepared as described above. Cells (2×10^7/ml) in PBS were stained with 1.25 μ*M* carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Molecular Probes, Eugene, OR) for 8 minutes. Stained cells were cultured with 25 μg/ml of recombinant human GPI at 1×10^6/ml in 96-well round-bottomed plates (Nunc) for 60 hours and then analyzed by flow cytometry.

Statistical analysis. Data are expressed as the mean ± SEM or mean ± SD. Differences between groups were examined for statistical significance using the Mann-Whitney U test. *P* values less than 0.05 were considered significant.

RESULTS

Induction of severe symmetric polyarthritis by immunization with GPI. For the induction of arthritis, we immunized DBA/1 mice with 300 μg of recombinant human GPI emulsified with Freund's complete adjuvant. Of the 177 mice immunized with recombinant human GPI, 167 (94.4%) developed severe swelling of the wrist and ankle joints (Figure 1A). The arthritis appeared on days 7–8, showed peak severity on day 14, then gradually subsided (Figure 1B). AQ: 7 F1

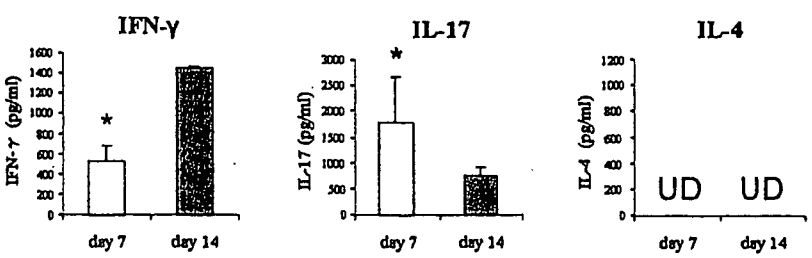

Figure 2. Differentiation of glucose-6-phosphate isomerase (GPI)–specific CD4+ T cells into Th1 and Th17 cells. CD4+ T cells and mitomycin C–treated antigen-presenting cells were stimulated for 24 hours with GPI on either day 7 (induction phase) or day 14 (effector phase) and then assessed for the production of interferon-γ (IFNγ), interleukin-17 (IL-17), and IL-4 by enzyme-linked immunosorbent assay. Values are the mean and SD of 3 independent experiments (n = 3 mice per experiment). * = $P < 0.05$ versus cells stimulated on day 14, by Mann-Whitney U test. UD = undetectable (<2 pg/ml).

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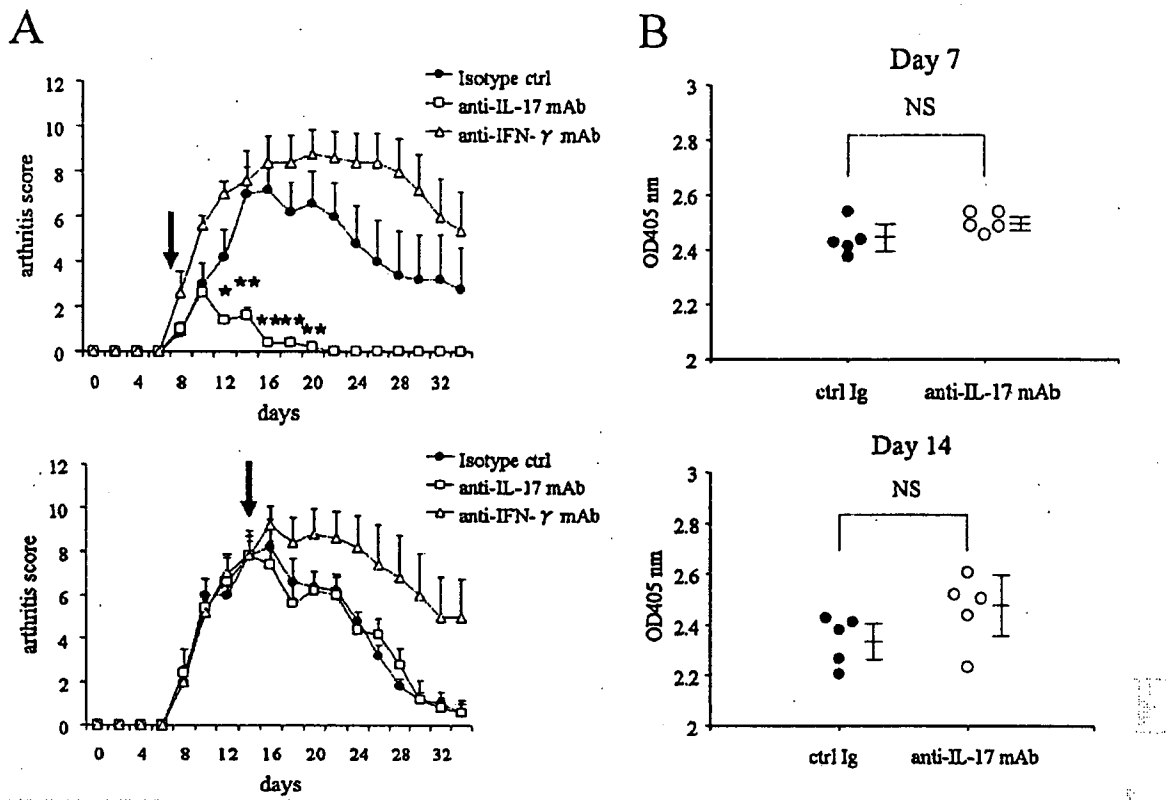

Figure 3. Suppression of the development of glucose-6-phosphate isomerase (GPI)–induced arthritis by treatment with by anti–interleukin-17 (anti–IL-17) monoclonal antibody (mAb). A, Arthritis scores following intraperitoneal injection of 100 μg of anti–IL-17 mAb or anti–interferon-γ (anti-IFNγ) mAb on day 7 or day 14 after GPI immunization (arrow). Values are the mean and SEM of 5 mice per group. Results are representative of 2 independent experiments. * = $P < 0.05$; ** = $P < 0.01$ versus isotype control at the same time point, by Mann-Whitney U test. B, Titers of anti-GPI antibody in sera obtained on day 35 following intraperitoneal injection of 100 μg of anti–IL-17 mAb on day 7 or day 14 after GPI immunization, as determined by enzyme-linked immunosorbent assay. Each symbol represents a single mouse. Bars show the mean ± SD optical density (OD) at 405 nm. NS = not significant (by Mann-Whitney U test).

Differentiation of GPI-specific CD4+ effector T cells to Th1 and Th17 cells, but not Th2 cells. CD4+ T cells are indispensable for both the induction phase and the effector phase of GPI-induced arthritis (3); however, the lineage to which GPI-specific CD4+ effector T cells are differentiated remains to be elucidated. To determine the lineage, we stimulated CD4+ T cells withrecombinant human GPI on day 7 (induction phase) or day 14 (effector phase) in vitro and then assessed cytokine production by ELISA. GPI-specific CD4+ T cells produced IFNγ and IL-17, but not IL-4, on days 7 and 14 (Figure 2). Interestingly, IFNγ production was lower on day 7 than on day 14 ($P < 0.05$), whereas IL-17 production was higher on day 7 than on day 14 ($P < 0.05$). These data demonstrated that GPI-specific CD4+ effector T cells are differentiated to Th1 and Th17 and are regulated differently during the development of arthritis.

Crucial role of Th17 cells in the induction phase. If GPI-specific CD4+ T cells produce both IFNγ and IL-17, then which of these two cytokines affects the development of arthritis? To answer this question, we injected 100 μg of anti-IFNγ mAb or anti–IL-17 mAb intraperitoneally on day 7 or day 14 after immunization with recombinant human GPI. Injection of anti–IL-17 mAb on day 7 resulted in significant improvement in the arthritis scores as compared with injection of isotype control ($P < 0.05$ or $P < 0.01$), but injection of anti–IL-17 mAb on day 14 did not affect the course of the disease (Figure 3A). In contrast, injection of anti-IFNγ mAb on day 7 and day 14 did not ameliorate arthritis, but rather, tended to exacerbate it (Figure 3A).

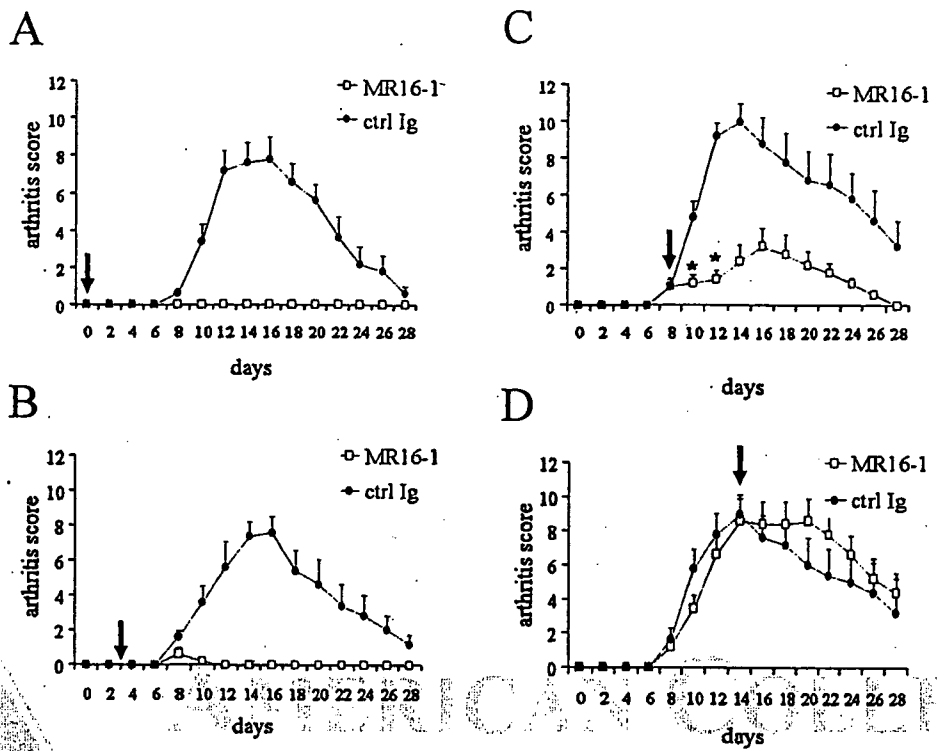

Figure 4. Inhibition of the development of arthritis by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 or control Ig on day 0 (A), day 3 (B), or day 8 (C) or with 4 mg of MR16-1 or control Ig on day 14 (D) after GPI immunization. The development of arthritis was monitored visually and scored on a scale of 0–3 (see Materials and Methods for details). Arrow indicates the date of mAb injection. Values are the mean and SEM of 5 mice per group. Results are representative of 2 independent experiments. * = $P < 0.05$ by Mann-Whitney U test.

Next, we explored whether anti–IL-17 mAb affects the production of anti-GPI antibodies. Treatment of mice with anti–IL-17 mAb on day 7 or on day 14 did not appreciably affect the titers of anti-GPI antibody (Figure 3B). These results indicate that Th17 cells are involved in the development of GPI-induced arthritis independently of anti-GPI antibody titers.

Inhibition of arthritis by anti–IL-6R mAb. It has been reported that IL-6 plays an important role in the differentiation of Th17 cells from naive T cells (8,9). We speculated that blockade of IL-6 might inhibit the development of arthritis, and we examined the effects of anti–IL-6R mAb MR16-1 on the development of arthritis. We injected 2 mg of MR16-1 intraperitoneally on day 0, 3, or 8 after immunization with recombinant human GPI, or we injected 4 mg on day 14 after immunization. As we anticipated, injection of MR16-1 on day 0 completely blocked the development of arthritis (Figure 4A), and injection on day 3 showed an almost complete inhibition (Figure 4B). Even after the development of arthritis, injection of MR16-1 on day 8 significantly suppressed the progression of arthritis (Figure 4C); however, injection of 4 mg of MR16-1 on day 14, at the peak of arthritis, did not ameliorate arthritis (Figure 4D). These results suggest that blockade of IL-6R has protective effects and some therapeutic effects on GPI-induced arthritis.

Inhibition of the development of Th17 cells, without an increase Th1, Th2, or Treg cell populations, by anti–IL-6R mAb. To examine whether MR16-1 affects Th1, Th2, and Treg cells, we cultured cells from draining lymph nodes obtained on day 7 in the presence of recombinant human GPI for 24 hours. Since the majority of cells that produce IL-17 are of the $CD4^{high}$ population, we analyzed IFNγ and IL-4 production gating on the $CD4^{high}$ population. We found that the majority of cells that produced cytokines such as IL-17 expressed $CD4^{high}$ cells. (An illustration of the data

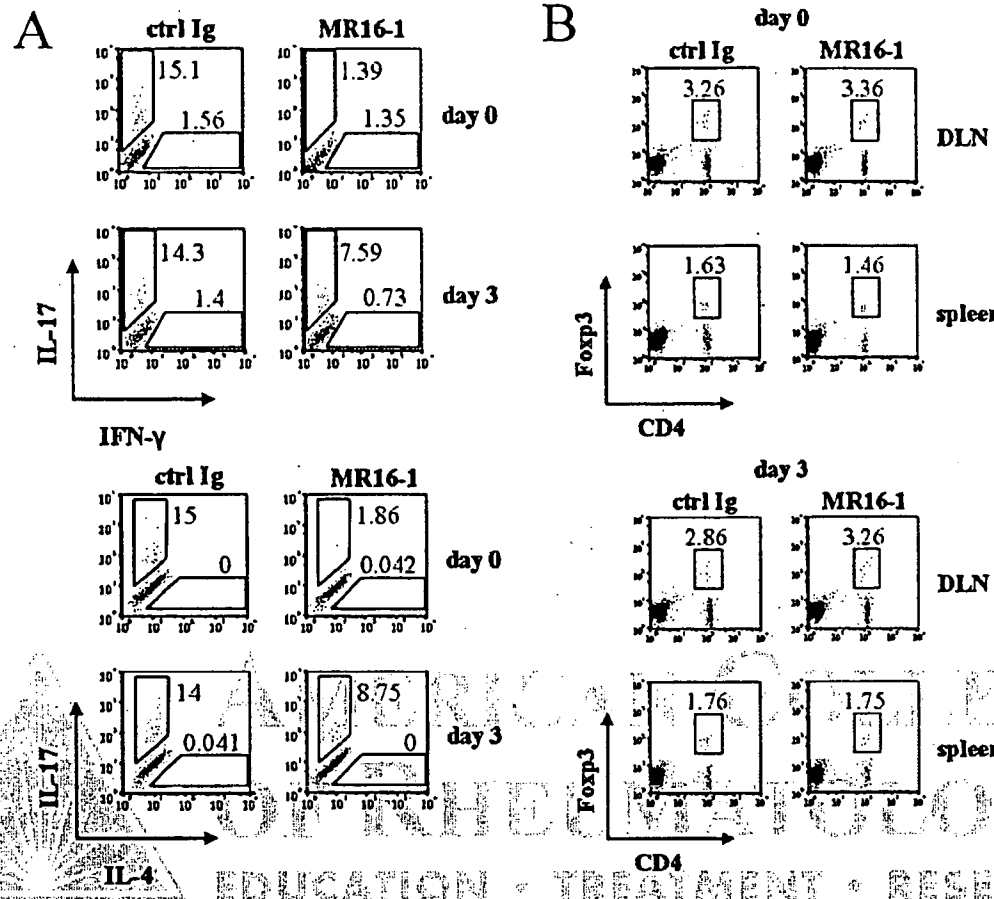

Figure 5. Inhibition of the differentiation of draining lymph node cells into Th17 cells by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 or with rat IgG (control) on day 0 or day 3 after GPI immunization. A, Cells from draining lymph nodes obtained on day 7 were cultured in the presence of 100 μg of recombinant human GPI. GoldiStop was added during the last 2 hours of each culture, and flow cytometric analysis of IL-17 and either interferon-γ (IFNγ) or IL-4 was performed, gating on $CD4^{high}$ cells. Results are representative of 3 independent experiments (n = 2 mice per experiment). B, Cells from draining lymph nodes (DLN) and spleen obtained on day 7 were stained with forkhead box P3 (FoxP3) and flow cytometric analysis of FoxP3 and CD4 was performed. Results are representative of 3 independent experiments (n = 2 mice per experiment). Values shown in the histograms are the percentages of positive cells in the compartment.

obtained from this analysis is available upon request from the corresponding author.)

We performed intracellular cytokine staining for IL-17, IFNγ, and IL-4 without nonspecific stimulants, such as phorbol myristate acetate or ionomycin, to assess physiologic cytokine production. Injection of MR16-1 on day 0 resulted in a significant decrease in IL-17 production by $CD4^{high}$ T cells (1.39%) as compared with injection of control Ig (15.1%) ($P < 0.05$), and there was a similar tendency with injection on day 3 (7.59% versus 14.3%; $P < 0.05$) (Figure 5A). IFNγ production was not significantly increased by MR16-1 injection on day 0 (1.35% versus 1.56%) or on day 3 (0.73% versus 1.4%) (Figure 5A). There was no difference in IL-4 production (Figure 5A). (A further illustration of the data obtained from this analysis is available upon request from the corresponding author.)

We also used intercellular staining methods to examine forkhead box P3 (FoxP3) expression after treatment with MR16-1. FoxP3-positive CD4+ T cells were essentially unaffected by MR16-1 treatment on day 0 or day 3 (Figure 5B). These data indicate that MR16-1 prevents the differentiation of naive T cells to Th17 cells, but does not affect other cell lineages.

Inhibition of the production of antigen-specific antibodies and antigen-specific proliferation of CD4+ T cells by anti–IL-6R mAb. We next explored whether MR16-1 affects the production of anti-GPI antibodies. Treatment of mice with MR16-1 resulted in significant reductions of anti-GPI antibody titers on days 3, 8, and 14 ($P < 0.0283$, $P < 0.0090$, $P < 0.0283$, respectively) as compared with mice injected with control Ig (Figure 6A). These results emphasize the inhibitory effects of MR16-1 on the production of anti-GPI antibodies irrespective of the phase of arthritis when treatment is administered.

In addition to antibody production, IL-6 is involved in T cell proliferation (10). Therefore, we explored whether MR16-1 affects antigen-specific proliferation of CD4+ T cells. Mice were injected intraperitoneally with 2 mg of MR16-1 on day 0, 3, or 8 after immunization of recombinant human GPI. Popliteal lymph nodes were harvested on day 10, cells stained with CFSE-DA were cultured with recombinant human GPI for 60 hours, and cell proliferation was analyzed by flow cytometry. As expected, CD4+ T cells treated with MR16-1 in vivo proliferated significantly less than those treated with control IgG (21.7% versus 2.22% on day 0, 30.3% versus 20.2% on day 3, 36.2% versus 27.7% on day 8) ($P < 0.05$) (Figure 6B). These data suggest that MR16-1 inhibits antigen-specific proliferation of CD4+ T cells, leading to a reduced population of antigen-specific CD4+ T cells in draining lymph nodes.

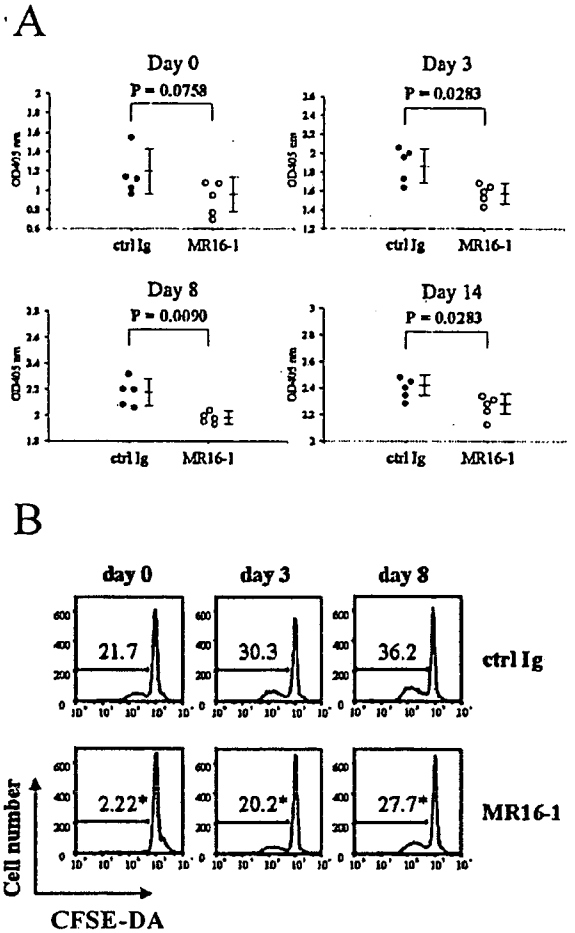

Figure 6. Inhibition of the production of anti–glucose-6-phosphate isomerase (anti-GPI) antibodies and the proliferation of CD4+ T cells by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). A, Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of mAb MR16-1 or rat IgG (control) on day 0, 3, or 8, or with 4 mg of mAb MR16-1 or control Ig on day 14 after GPI immunization. Sera were obtained on day 28, and the titers of anti-GPI antibodies were analyzed by enzyme-linked immunosorbent assay. Each symbol represents a single mouse. Bars show the mean ± SD optical density (OD) at 405 nm. *P* values were determined by Mann-Whitney U test. B, Mice were injected intraperitoneally with 2 mg of mAb MR16-1 or rat IgG (control) on day 0, 3, or 8 after immunization. Cells from draining lymph nodes (DLN) obtained on day 10 were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE-DA), cultured with 25 μg of recombinant human GPI for 60 hours, and cell proliferation was analyzed by flow cytometry. Values are the percentage of proliferating cells. Results are representative of 3 independent experiments (n = 2 mice per experiment). * = $P < 0.05$ versus controls, by Mann-Whitney U test.

DISCUSSION

GPI, a ubiquitous glycolytic enzyme, is a new candidate autoantigen in the initiation of autoimmune arthritis (11). The arthritogenicity of GPI was first described in T cell receptor–transgenic K/BxN mice (2). In K/BxN mice, CD4+ T cells (especially KRN T cells) were required for the development of arthritis, although they appeared to be dispensable after the mice produced arthritogenic autoantibodies to GPI (12). While the K/BxN mouse is a striking model of spontaneous arthritis, the effectiveness of biologic agents used to treat the arthritis is limited. Tumor necrosis factor α (TNFα) blockade had no effect on the development and progression of arthritis in K/BxN mice (12), and serum transfer from arthritic K/BxN mice into IL-6–deficient mice did not affect the course of arthritis as compared with that in wild-type mice (13).

GPI-induced arthritis is produced by immunization of genetically unaltered DBA/1 mice with GPI. In GPI-induced arthritis, administration of either anti-TNFα mAb or CTLA-4Ig after the onset of arthritis shows a significant amelioration of the arthritis (Matsumoto I, et al: unpublished observations). This model is different from the CIA model, in that GPI-induced

arthritis is T cell–dependent. In GPI-induced arthritis, administration of anti-CD4 mAb around the time of immunization was shown to completely prevent arthritis, and more noteworthy, administration of anti-CD4 mAb on day 11 and on day 14 was shown to induce rapid remission of the arthritis (3). These findings highlight the importance of CD4+ T cells in the induction phase and the effector phase of GPI-induced arthritis. In contrast, in CIA, CD4+ T cells are indispensable only until the B cells produce autoantibodies, since anti-CD4 mAb treatment is ineffective when administered after anti-GPI antibodies have appeared (4,5). Judging from these findings, GPI-induced arthritis is considered a useful murine model for analyzing the role of CD4+ T cells in the effector phase of the arthritis.

Several studies have examined the roles of Th17 cells, a distinct lineage of CD4+ effector T cells, in various arthritis models (14–17). CIA was shown to be partially suppressed in IL-17–deficient mice (16), whereas it was exacerbated in IFNγ-deficient mice or IFNγ receptor–deficient mice (18–20). Despite of the similarity of Th1 and Th17, the efficacy of anti–IL-17 mAb treatment in GPI-induced arthritis was more marked than in CIA. In the CIA model, administration of anti–IL-17 antibodies during the induction phase of arthritis was shown to only partially inhibit the development of arthritis (21). This difference between GPI-induced arthritis and CIA may reflect a more substantial contribution from cells of the Th17 lineage. In our experiments, the production of IL-17 on day 7 was higher than that on day 14, and for IFNγ, the inverse was true, with lower production of IFNγ on day 7 than on day 14. It has been reported that IFNγ suppresses the production of IL-17 by inhibiting IL-23R (22,23); therefore, a cytokine milieu in which little IFNγ is present during the induction phase of arthritis might boost the production of a large amount of IL-17, and conversely, a milieu in which large amounts of IFNγ are present during the effector phase of arthritis might inhibit the production of IL-17. This might also account for the fact that spontaneous remission began on day 14 in mice with GPI-induced arthritis.

Recent in vitro studies indicated that IL-6 is an essential inducer of the differentiation of Th17 cells (8,9). In our experiments, blockade of IL-6R on days 0 and 3 markedly suppressed the production of IL-17 and the proliferation of GPI-specific CD4+ T cells in vivo. In contrast, GPI-induced arthritis was suppressed by MR16-1 administration on days 0 and 3, and when MR16-1 was administered on day 8, the arthritis was ameliorated, which likely occurred through inhibition of T cell proliferation and autoantibody production, rather than blockade of Th17 differentiation. MR16-1 also suppressed autoantibody production most significantly when administered on day 8. This effect was probably mediated through a direct action on B cells (24,25) because the production of anti-GPI antibodies was highest around day 8 (Matsumoto I, et al: unpublished observations).

In the present experiments, the dose of MR16-1 we administered was 20–40 times higher than the dose of the anti–IL-17 mAb. MR16-1 is a mAb against murine IL-6R, and for there to be sufficient inhibition of the biologic activity of IL-6 in vivo, soluble IL-6 receptors, which are consistently present in the blood, would have to be blocked. Therefore, a relatively high dose would be needed compared with the titer of antibodies to the cytokine itself. This idea is supported by our unpublished data (Matsumoto I, et al: unpublished observations) showing that MR16-1 inhibited the biologic activity of IL-6 in vitro when administered at the same concentration as other antibodies to the cytokine itself.

Are these scenarios applicable to RA in humans? The therapeutic effects of a humanized anti–IL-6Rα antibody (tocilizumab) on RA have recently been reported (26,27). Patients with severe forms of RA retained high titers of anti-GPI antibodies (7,28,29), although a few control subjects also had these antibodies. In anti-GPI antibody–positive individuals, GPI-reactive CD4+ T cells, especially Th1-type cells, were specifically detected in peripheral blood mononuclear cells from RA patients who shared either the HLA-DR*0405 or *0901 haplotype (30). What about mice with GPI-induced arthritis? High titers of anti-GPI antibodies have been found to be produced by arthritis-resistant C57BL/6 mice as well, although their T cells exhibited weak GPI responses (ref. 3 and Matsumoto I, et al: unpublished observations) as compared with the responses of T cells from arthritis-susceptible DBA/1 mice.

These findings indicate that anti-GPI antibodies are not sufficient for the induction of arthritis; it is probable that the support of antigen-specific T cell activation is indispensable. In this regard, GPI-induced arthritis seems to be a useful model for analyzing the pathology of RA in humans. In addition, it has been shown that TNF antagonists clearly inhibit the progression of GPI-induced arthritis (3), even after clinical onset of disease (Matsumoto I, et al: unpublished observations). In our present study, administration of anti–IL-17 mAb or MR16-1 on day 14 (late effector phase) was not able to ameliorate GPI-induced arthritis. However, both the IL-6/IL-17 axis and TNFα might play a crucial role in established RA, since both tocilizumab and TNF antagonists have shown marked therapeutic

efficacy in humans with established RA (26,27,31–34), although administration of MR16-1 or anti-TNF mAb has shown no effect or only a weak effect on fully established CIA in mouse models (35,36). Further analysis is necessary to determine whether GPI-reactive Th17 cells exist in the peripheral blood or joints of patients with RA who have anti-GPI antibodies.

In conclusion, the findings of our study highlight the importance of the IL-6/IL-17 axis in GPI-induced arthritis, a murine model of RA. Blockade of IL-6R might be a useful therapeutic strategy in Th17-mediated arthritis. Since a humanized anti–IL-6R mAb has been shown to have an excellent therapeutic effect on RA, further studies are needed to confirm that the IL-6/IL-17 axis is also crucial in RA.

AUTHOR CONTRIBUTIONS

AQ: 23 Dr. Matsumoto 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 design. Iwanami, Matsumoto, Sumida.

Acquisition of data. Iwanami, Matsumoto, Tanaka-Watanabe, Inoue, Mihara, Ohsugi, Mamura, Goto, Ito, Tsutsumi, Kishimoto, Sumida.

Analysis and interpretation of data. Iwanami, Matsumoto, Sumida.

Manuscript preparation. Iwanami, Matsumoto, Sumida.

Statistical analysis. Iwanami, Matsumoto.

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High prevalence of autoantibodies to muscarinic-3 acetylcholine receptor in patients with juvenile-onset Sjögren syndrome

Sjögren syndrome (SS) is an autoimmune disease characterised pathologically by lymphocytic infiltration into the lacrimal and salivary glands, and clinically by dry eyes and mouth. Lymphocytic infiltration is also found in the kidneys, lungs, thyroid, and liver. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lacrimal glands and the kidneys are CD4-positive αβT cells.[1] Candidate autoantigens recognised by T cells that infiltrate the labial salivary glands of SS have been analysed and Ro/SS-A 52 kDa,[2] α-amylase, heat shock protein, and TCR BV6[3] have been identified, although Ro/SS-A 52 kDa reactive T cells were not increased in peripheral blood.[4]

In contrast, various autoantibodies (autoAbs) have been identified in the sera of patients with SS, and some of these autoAbs, such as anti-SS-A antibody (Ab) and anti-SS-B Ab, are used as diagnostic markers. Muscarinic-3 acetylcholine receptor (M3R) is involved in activation of salivary and lacrimal glands. This receptor is G-protein-linked and its activation triggers a second-messenger cascade that culminates in a rise in intracellular calcium and activation of K^+ and Cl^- channels that drive fluid secretion.[5] Although autoAbs to M3R have been demonstrated in patients with SS,[6] the location of B cell epitopes on M3R remain controversial.[7 8] We previously reported the presence of autoAbs against the second loop domain of M3R in 11.2% of patients with adult SS.[9] Anti-M3R Ab is specific for SS because it is not present in patients with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythaematosus. Based on these early findings, we hypothesised that the presence of anti-M3R Ab may be directly related to defective salivary and lacrimal secretion in SS patients. The prevalence of M3R Ab in juvenile SS is still unknown. To examine this issue, we screened sera of patients with juvenile SS for anti-M3R Ab.

Serum samples were collected from 38 Japanese paediatric patients with juvenile-onset SS (JSS) followed-up at the Departments of Pediatrics of Graduate School of Medicine, Chiba University and Yokohama City University School of Medicine, Yokohama. We recruited 76 healthy control subjects from the Division of Clinical Immunology, Major of Advanced Biological Applications, Graduate School Comprehensive Human Science, University of Tsukuba. The mean (SD) age of the patients was 15 (5) and 22 (2) years for the JSS and control groups, respectively. The 25mer synthetic amino acid encoding the second extracellular domain of M3R was used as the antigen, because this portion plays an important role in intracellular signalling. Figure 1 shows that the mean titre of anti-M3R Ab in patients with JSS (0.329 (0.189)) was significantly higher than that of controls (0.105 (0.089), $p<0.001$). Moreover, the prevalence of anti-M3R Ab in patients with JSS (52.6%) was significantly higher than that

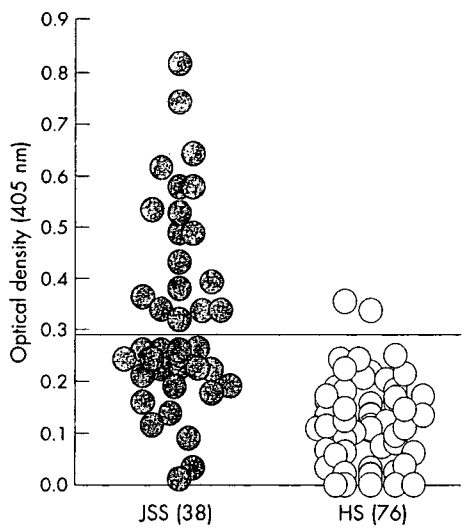

Figure 1 Comparison of anti-M3R Abs in patients with juvenile Sjögren syndrome (JSS) and control. A 25mer peptide (KRTVPPGECFIQFLSEPTITFGTAI) corresponding to the sequence of the second extracellular loop domain of the human M3R was synthesised (Sigma-Aldrich Japan, Ishikari, Japan). A 25mer peptide (SGSGSGSGSGSGSGSGSGSGSGS) was also synthesised as a negative control (Sigma-Aldrich Japan). Peptide solution (100 μl/well at 10 μg/ml) in 0.1M Na_2CO_3 buffer, pH 9.6, was adsorbed onto a Nunc-Immuno plate (Nalge Nunc International, Rochester, New York, USA) overnight at 4°C, and blocked with 5% bovine serum albumin (Wako Pure Chemical Industries, Osaka, Japan) in phosphate buffered saline (PBS) for 1 h at 37°C. Serum at 1:50 dilution in blocking buffer was incubated for 2 h at 37°C. The plates were then washed twice with 0.05% Tween 20 in PBS, and 100 μl of alkaline phosphatase-conjugated goat antihuman IgG (Fc; American Qualex, San Clemente, California, USA) diluted 1:000 in PBS was added for 1 h at room temperature. After three washes, 100 μl of *p*-nitrophenyl phosphate (Sigma) solution (final concentration 1 mg/ml) was added as alkaline phosphate substrate. Plates were incubated for 30 min at room temperature and the optical density at 405 nm was measured by plate spectrophotometry (Bio-Rad Laboratories, Hercules, California, USA). Optimal density was used to express the titre of anti-M3R Abs. Measurements were performed in triplicate and standardised between experiments. Numbers in parentheses represent the number of patients in each group.

in controls (2.9%, p<0.001). These results indicate the high prevalence of anti-M3R in JSS patients, compared to adult-onset SS patients. The presence of anti-SS-A Ab or anti-SS-B Ab were not associated with the presence of anti-M3R Ab in patients with JSS.

In conclusion, the high titre and prevalence of anti-M3R Abs in patients with JSS suggest that anti-M3R Ab could be potentially useful as a diagnostic marker for JSS.

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Association of the *IFIH1-GCA-KCNH7* chromosomal region with rheumatoid arthritis

The cause of rheumatoid arthritis (RA) remains unknown, although both genetic and environmental factors are involved. A genome-wide association study of non-synonymous single nucleotide polymorphisms (SNPs) showed the involvement of rs1990760, a change Ala946Thr in the *IFIH1* interferon induced helicase gene, in type 1 diabetes risk.[1] Further analyses of the locus surrounding this marker obtained compelling statistical support of the genetic equivalence of some other SNPs in strong linkage disequilibrium with rs1990760, making it impossible to ascertain the aetiological variant. The associated chromosomal region in 2q24.3 includes three genes: *IFIH1*, also known as *helicard* or melanoma differentiation associated gene-5 (*mda-5*)[2]; grancalcin (*GCA*); and a potassium voltage gated channel (*KCNH7*). We analysed three variants along this region to investigate whether this locus is involved in RA, another autoimmune disease.

We replicated the effect of the *IFIH1* polymorphism previously associated with type 1 diabetes in our RA cohort (to locate genes and polymorphisms, see supplementary fig 2 in Smyth *et al*[1]). Two flanking variants were studied and a significant protection was also observed for an intronic polymorphism of the *KCNH7* gene (table 1). The third polymorphism, rs13422767, departed from Hardy–Weinberg equilibrium in our control cohort, so no sound conclusion can be drawn from its analysis. Stratification caused by the presence of the shared epitope (SE), the main genetic risk factor in RA, yielded no significant difference between SE positive and SE negative patients.

Consecutively recruited Spanish RA patients (69% women) and ethnically matched healthy controls (51% women) were included in a case–control study approved by the Hospital Clínico, Madrid. The RA diagnosis was established based on the American College of Rheumatology (ACR) criteria. Mean (SD) age at onset was 53 (14) years; 59% patients carried the shared epitope, 75% were positive for rheumatoid factor, and 50% had anti-CCP antibodies. Genotyping was carried out by using TaqMan assays under the conditions recommended by the manufacturer (Applied Biosystems, Foster City, California, USA). Statistical analyses were done using standard statistical software (SPSS v12.0).

The three genes located in this locus are potential candidates for involvement in autoimmune diseases. Viral agents have been implicated in the aetiology of diverse chronic autoimmune disorders, RA included.[3 4] Although the causal link cannot be unequivocally supported, viruses may participate in the progression or exacerbation of inflammatory responses within the RA joint.[5] Specific receptors of the innate immune system detect the presence of viruses, promoting the production of proinflammatory cytokines. One of these receptors is the RNA

Table 1 Genotype frequencies of three single nucleotide polymorphisms in the 2q24.3 chromosomal region containing the *IFIH1-GCA- KCNH7* genes

	Upstream *IFIH1*, rs13422767 (%)			*IFIH1*, rs1990760 (%)			*KCNH7*, rs2068330 (%)		
	GG	GA	AA	AA	AG	GG	CC	CG	GG
Controls (n = 535)	377 (70)	136 (25)	22 (4)	188 (35)	254 (47)	93 (17)	204 (38)	252 (45)	79 (16)
RA patients (n = 540)	397 (74)	127 (23)	16 (3)	222 (41)	235 (43)	83 (15)	238 (44)	244 (45)	58 (10)
SE positive (n = 271)	201 (74)	62 (23)	8 (3)	107 (40)	123 (45)	41 (15)	120 (44)	125 (46)	26 (10)
SE negative (n = 193)	138 (72)	46 (24)	9 (4)	79 (41)	79 (41)	35 (18)	80 (42)	86 (45)	27 (14)

RA *vs* controls: rs1990760 G *vs* A, p = 0.058; rs2068330 G *vs* C, p = 0.016 (odds ratio (95% confidence interval) = 0.85 (0.71 to 1.01) and 0.8 (0.67 to 0.96)).
RA, rheumatoid arthritis; SE, shared epitope.

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Effects of Infliximab Therapy on Gene Expression Levels of Tumor Necrosis Factor α, Tristetraprolin, T Cell Intracellular Antigen 1, and Hu Antigen R in Patients With Rheumatoid Arthritis

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Objective. **Tristetraprolin (TTP), T cell intracellular antigen 1 (TIA-1), and Hu antigen R (HuR) are adenine/uridine-rich element binding proteins (ABPs) that affect the production of tumor necrosis factor α (TNFα) by binding to TNF messenger RNA (mRNA). TTP promotes deadenylation, TIA-1 inhibits translation, and HuR stabilizes TNFα mRNA. The aims of this study were to understand the posttranscriptional control of TNFα production in patients with rheumatoid arthritis (RA), and to identify parameters that may predict the efficacy of anti-TNFα therapy.**

Methods. **Peripheral blood mononuclear cells from 38 patients with RA were obtained before therapy and 2 weeks and 54 weeks after administration of the first dose of infliximab, and from 20 healthy control subjects. TNFα, TTP, TIA-1, and HuR gene expression levels were analyzed by real-time polymerase chain reaction.**

Results. **At baseline, TTP and HuR gene expression levels, as well as the TTP:TNFα, TTP:HuR, and TIA-1:TNFα gene expression ratios were lower in patients with RA than in control subjects, while expression of TNFα, TIA-1, and TIA-1:HuR was higher in patients with RA. The TTP:HuR expression ratio decreased significantly after administration of infliximab. Positive correlations were observed between TNFα and TTP, TNFα and TIA-1, TIA-1 and HuR, and TNFα and HuR gene expression in both healthy control subjects and patients with RA. At baseline, the TIA-1:HuR ratio tended to be higher in patients who achieved 50% improvement according to the American College of Rheumatology criteria (ACR50) at week 54 than in those who did not achieve at least an ACR20 response.**

Conclusion. **Differences in ABP gene expression may affect TNFα gene expression. A higher TIA-1:HuR expression ratio might correlate with the response to infliximab therapy.**

Rheumatoid arthritis (RA) is a relatively common chronic systemic inflammatory disease, affecting nearly 1% of the world's population (1). Although the pathogenesis of RA is not fully understood, tumor necrosis factor α (TNFα) is one of the most important cytokines involved in the development of synovitis (2–4). TNFα is produced by activated macrophages, lymphocytes, and synovial cells and induces other proinflammatory agents, including interleukin-1α (IL-1α), IL-6, and IL-15. All of these cytokines are involved in synovial cell activation and proliferation, leading to pannus formation in the joints (5–8). They also enhance the synthesis and action of proteases such as metalloproteinases, eventually causing cartilage and bone destruction (5). Antagonists to these cytokines, such as infliximab, etanercept, adalimumab (TNFα antagonists), and tocilizumab (IL-6 antagonist), are effective in relieving these cytokine-induced symptoms of RA in individual patients (9–21). In fact, the beneficial effects of TNFα antago-

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nists confirm the central role of TNFα in the inflammatory process of RA. However, some patients do not respond to TNFα antagonists, and it is not currently possible to predict the efficacy of these drugs prior to therapy. Thus, a better understanding of the mechanisms that control TNFα production is needed to develop new therapies and to envision the responses of individual patients to anti-TNFα therapy.

Production of TNFα is regulated both transcriptionally and posttranscriptionally. Degradation of messenger RNA (mRNA) regulated by adenine/uridine-rich elements (AREs), which are present in the 3′-untranslated region of transcripts encoding inflammatory cytokines such as TNFα, is a paradigm for posttranscriptional regulation (22–24). ARE-binding proteins (ABPs) that can affect the production of cytokines and other inflammatory molecules have been identified (25). Of these, tristetraprolin (TTP), T cell intracellular antigen 1 (TIA-1), and Hu antigen R (HuR) are the most studied to date (26–42).

TTP is a widely expressed protein with 2 zinc finger domains that act as active RNA binding sites. TTP is an immediate early response gene expressed in fibroblasts and other cells upon induction by a variety of stimuli (29–32). TTP binds to AREs of TNFα mRNA and promotes mRNA degradation, thereby reducing the production of TNFα (33). TTP-knockout mice display an inflammatory phenotype characterized by inflammatory arthritis, dermatitis, cachexia, autoimmunity, and myeloid hyperplasia; this phenotype can be prevented by administration of anti-TNFα antibodies (34).

TIA-1 contains 3 RNA recognition motifs (RRMs) that confer high-affinity binding to uridine-rich motifs (35). Recent studies have shown that upon binding to AREs, TIA-1 works not as a transcript destabilizer but as a translational silencer (28). Mild arthritis develops in TIA-1–knockout mice, and severe arthritis develops in TIA-1/TTP–double-knockout mice (36).

The other ABP, HuR, is a member of the embryonic lethal abnormal vision RNA-binding proteins and is ubiquitously expressed in proliferating cells (27). HuR has 3 RRMs that bind to ARE at the poly A tail of various mRNAs, and it participates in the regulation of ARE-mediated mRNA stabilization (37,38). Overexpression of HuR stabilizes mRNA-containing TNFα AREs, implicating TNFα AREs as a target for HuR (39). Although HuR gene–knockout mice have been not reported and our knowledge on HuR function is limited, HuR is assumed to accelerate the posttranscriptional production of TNFα by stabilizing its mRNA (41,42).

Recently, we reported that the TTP gene is overexpressed in synovial tissue from patients with RA compared with that from patients with osteoarthritis (OA) (40). Interestingly, when TTP and TNFα gene expression was compared, synovial tissue from patients with elevated serum C-reactive protein (CRP) levels tended to have a low TTP:TNFα gene expression ratio. Thus, appropriate expression of the TTP gene may be important in reducing the severity of RA. This prompted us to speculate that the magnitude and balance of expression of these ABP genes are of importance in determining the severity of RA. Inadequate expression of these genes may result in more severe disease or refractory responses to therapies including anti-TNFα agents. However, although measurement of gene expression in the joint synovium is informative, it would be impossible to obtain clinical samples at the desired time points for adequate monitoring of the disease activity or drug efficacy. It is also almost impossible to obtain samples from healthy control subjects.

The aims of this study were to understand the posttranscriptional control of TNFα production in RA and to identify parameters that could predict the efficacy of anti-TNFα therapy. For this purpose, we measured gene expression of TNFα, TTP, HuR, and TIA-1 in peripheral blood mononuclear cells (PBMCs) from patients with RA. The samples were obtained at baseline and 2 weeks and 54 weeks after administration of the first dose of the anti-TNFα monoclonal antibody, infliximab, and were compared among each other and with those obtained from healthy control subjects.

PATIENTS AND METHODS

Patients. Thirty-eight patients with RA (15 men and 23 women, mean ± SD age 53.0 ± 11.5 years) and 20 healthy control subjects (14 men and 6 women, mean ± SD age 31.9 ± 8.40 years) were included in this study. All patients fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 criteria for the classification of RA (43) and had active arthritis in spite of oral methotrexate therapy (at least 6 mg/week for more than 6 weeks). The characteristics of the participants are listed in Table 1. Written informed consent was obtained from all patients, and the study was approved by the appropriate ethics committee.

Infliximab therapy and assessment of efficacy. Patients were treated with 3 mg/kg of infliximab at weeks 0, 2, 6, and 14, and every 8 weeks thereafter. Infliximab efficacy was evaluated using the ACR preliminary criteria for improvement in RA (44), 54 weeks after the initiation of infliximab therapy. Patients who achieved 50% improvement (an ACR50 response) at week 54 (n = 14) were included in the responder group, while those with less than 20% improvement at week 54 (n = 9) were classified as nonresponders.

Table 1. Characteristics of the patients with RA and healthy controls*

Characteristic	RA patients	Healthy controls
Age, years	53.0 ± 11.5 (25–69)	31.9 ± 8.40 (20–52)
No. men/no. women	15/23	14/6
Disease duration, years	8.68 ± 5.74 (1.67–24)	–
C-reactive protein, mg/dl	3.21 ± 1.93 (0.31–7.77)	–
ESR, mm/hour	59.5 ± 22.1 (15–104)	–
Rheumatoid factor, IU/ml	227 ± 362 (5–1,790)	–
Methotrexate, mg/week	7.67 ± 1.31 (6–12)	–
Prednisolone, mg/day	7.03 ± 4.00 (0–17.5)	–

* Except where indicated otherwise, values are the mean ± SD (range). RA = rheumatoid arthritis; ESR = erythrocyte sedimentation rate.

Samples and complementary DNA (cDNA) synthesis. Peripheral blood was obtained from healthy control subjects and from patients with RA, before (week 0) and 2 weeks and 54 weeks after they received the first dose of infliximab. PBMCs were isolated from heparinized peripheral blood using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), following the protocol recommended by the manufacturer. Cells were spun down to pellets, and total RNA was extracted from the cell pellets using Isogen (Nippongene, Tokyo, Japan). Complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD), following the instructions provided by the manufacturer.

Quantification of gene expression by real-time polymerase chain reaction. The cDNA samples were amplified with specific primers and fluorescence-labeled probes for the target genes. Amplified product genes were monitored on an ABI 7700 Sequence Detection system (Applied Biosystems, Tokyo, Japan). qPCR MasterMix was purchased from Eurogentec (Seraing, Belgium). The final magnesium concentration was 5 n*M*, the final primer concentration was 200 n*M* for each 5′ and 3′ primer, and the final probe concentration was 100 n*M*. Primers and fluorescent probes for TNFα, TTP, TIA-1, HuR, and GAPDH were purchased from Applied Biosystems. Thermal cycler conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Serial dilutions of a standard sample were included in every assay, and standard curves for the genes of interest and the GAPDH gene were generated. All measurements were performed in triplicate. The level of gene expression was calculated from the standard curve and was expressed relative to GAPDH gene expression.

Statistical analysis. The Wilcoxon rank test for paired samples was used to compare the gene expression levels among samples obtained at week 0, week 2, and week 54. The Mann-Whitney U rank test was used to compare the expression levels of genes in patients with RA and healthy control subjects. Pearson's correlation coefficient was calculated to assess the correlations between the expression of 2 genes. All data are expressed as the mean ± SD. *P* values less than 0.05 were considered significant. Statistical analyses were performed using StatView version 5.0 software (SAS Institute, Cary, NC).

RESULTS

Expression levels of TNFα and ABP genes. At week 0 (baseline), TNFα gene expression in PBMCs was higher in patients with RA than in healthy control subjects (for patients with RA, mean ± SD 2.80 ± 2.48; for control subjects, 0.88 ± 0.46 [$P < 0.0001$]). In contrast, expression levels of the TTP gene were lower in patients with RA than in control subjects (1.20 ± 0.95 and 2.60 ± 1.54, respectively [$P < 0.0001$]). Expression levels of the TIA-1 gene were higher and those of the HuR gene were lower in patients with RA than in control subjects (for TIA-1, 3.34 ± 1.79 and 1.79 ± 0.39, respectively [$P < 0.0005$]; for HuR, 1.79 ± 0.83 and 2.15 ± 0.59, respectively [$P = 0.018$]) (Figure 1). When the expression levels of 2 genes in a given sample were compared, the TTP:TNFα, TTP:HuR, and TIA-1:TNFα ratios were significantly lower in patients with RA than in control subjects (for TTP:TNFα, 0.55 ± 0.43 and 3.09 ± 1.17, respectively [$P < 0.0001$]; for TTP:HuR, 0.90 ± 1.09 and 1.19 ± 0.53, respectively [$P < 0.005$]; for TIA-1:TNFα, 1.80 ± 1.42 and 2.40 ± 0.87, respectively [$P = 0.014$]), while the TIA-1:HuR gene expression ratio was significantly higher in PBMCs from patients with RA than in those from control subjects (1.85 ± 0.52 and 0.85 ± 0.14, respectively [$P < 0.0001$]) (Figure 1). Among these comparisons, the difference in the TTP:TNFα ratio appeared to be most prominent, and this significant difference may imply that TTP is important as a negative regulator of inflammation in RA.

TNFα and ABP gene expression levels before and after infliximab therapy. We compared the gene expression levels of TNFα, TTP, TIA-1, and HuR in PBMC samples obtained at baseline and 2 weeks and 54 weeks after administration of the first dose of infliximab. No significant differences were noticed between baseline and week 2 samples (for TNFα, 2.80 ± 2.48 at week 0