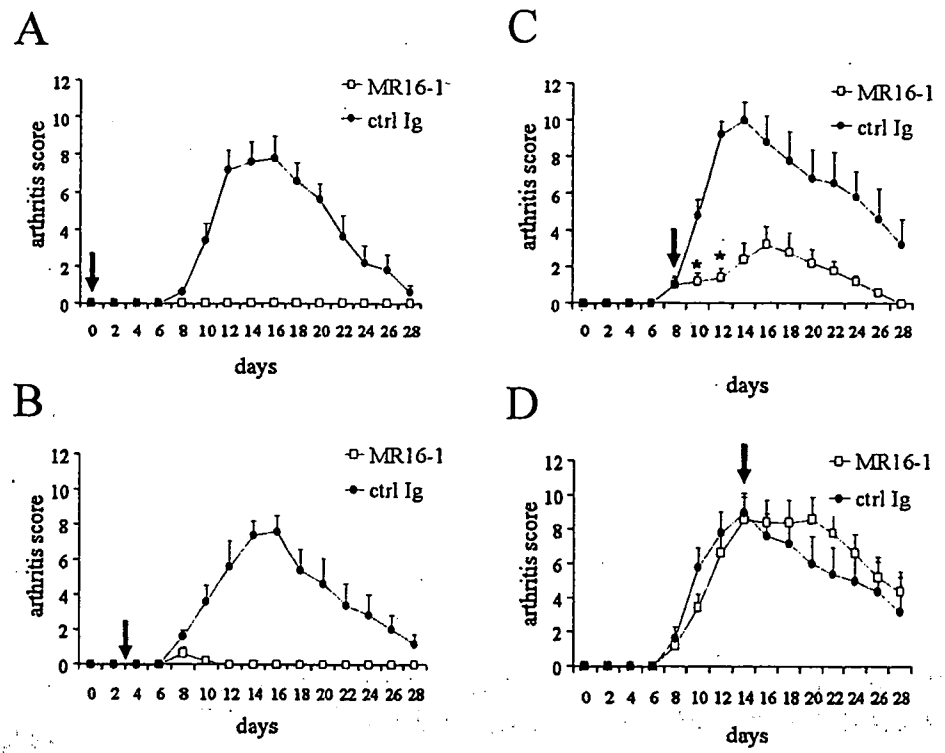

Figure 4. Inhibition of the development of arthritis by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 or control Ig on day 0 (A), day 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

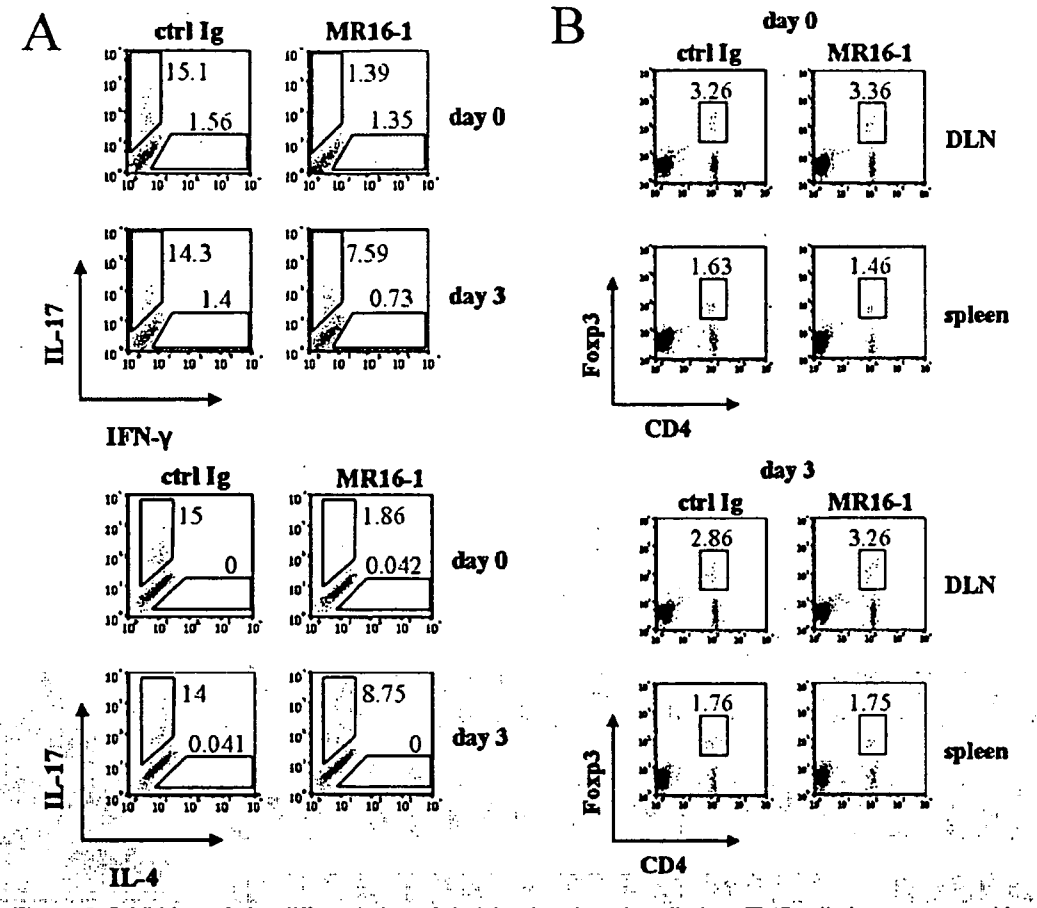

Figure 5. Inhibition of the differentiation of draining lymph node cells into Th17 cells by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 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.

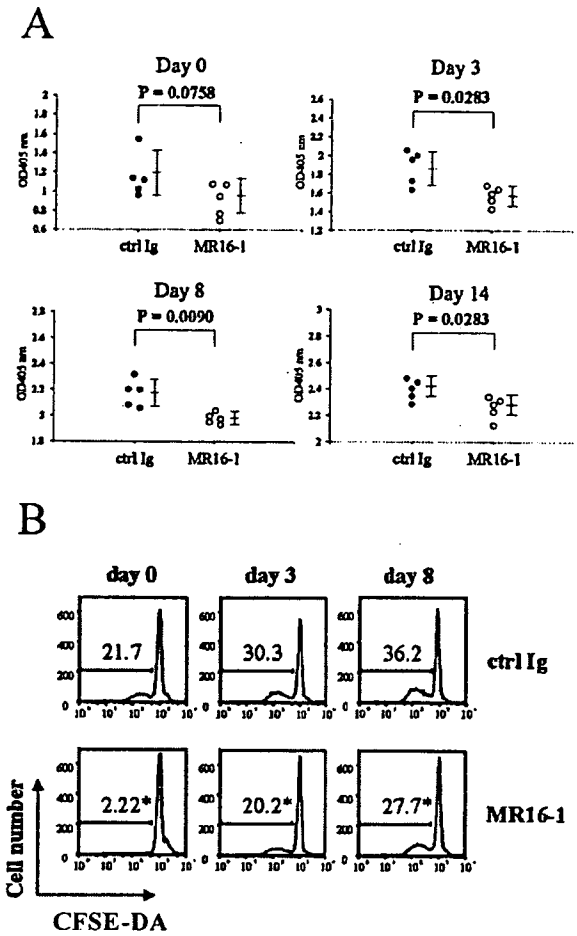

Figure 6. Inhibition of the production of anti–glucose-6-phosphate isomerase (anti-GPI) antibodies and the proliferation of CD4+ T cells by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). A, Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally 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

Q: 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

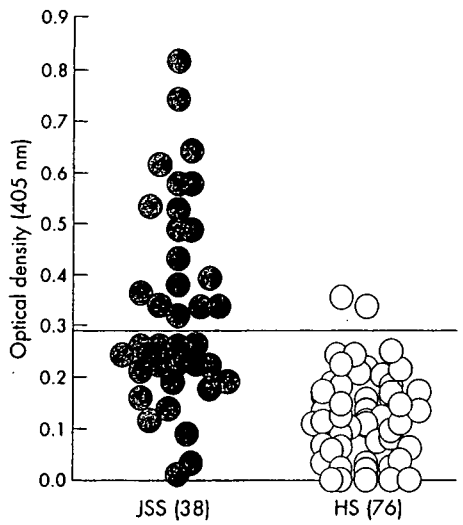

Figure 1 Comparison of anti-M3R Abs in patients with juvenile Sjögren syndrome (JSS) and control. A 25mer peptide (KRTVPPGECFIQFLSEPTITFGTAI) corresponding to the sequence of the second extracellular loop domain of the human M3R was synthesised (Sigma-Aldrich Japan, Ishikari, Japan). A 25mer peptide (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.

Biased usage of synovial immunoglobulin heavy chain variable region 4 by the anti-glucose-6-phosphate isomerase antibody in patients with rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA) is the most common inflammatory arthritis, characterized by marked infiltration of mononuclear cells including B cells into the inflamed synovium. Anti-glucose-6-phosphate isomerase (GPI) antibody (Ab) is an arthritogenic Ab in K/BxN T cell receptor transgenic mice, and is also present in some patients with RA. To characterize synovial B cells from anti-GPI Ab-positive RA, synovial immunoglobulin (Ig) heavy chain variable regions (VH) were compared with those of negative individuals. Synovial tissues were obtained from six RA patients (three anti-GPI Ab-positive and three anti-GPI Ab-negative). Ig-VH genes were amplified by PCR using family-specific primers and were subsequently sequenced. In synovial B cells from anti-GPI Ab-positive RA patients, VH4 and JH4 were predominantly expressed ($p<0.0001$). The immunoglobulin heavy chain complementarity-determining region 3 (IgH-CDR3) length in the synovium of anti-GPI Ab-positive individuals was shorter than that in anti-GPI Ab-negative individuals ($p=0.0005$). In addition, the IgH-CDR3 of anti-GPI Ab-positive patients was rich in basic-ionized amino acids (arginine, histidine, and lysine) near their central position, suggesting a high affinity. Our results support the notion that Ig-VH4 B cells in RA synovium with anti-GPI Ab are affinity-matured and that anti-GPI Ab might be associated with the skewed IgH-CDR3.

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Abbreviations: -, negative; +, positive; DH, heavy chain diversity regions; FR, framework region; GPI, glucose-6-phosphate isomerase; IgH-CDR3, immunoglobulin heavy chain complementarity determining region 3; JH, heavy chain joining regions; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; VH, heavy chain variable regions

Key words: autoantibodies, rheumatoid arthritis, glucose-6-phosphate isomerase, B cells, synovium

Introduction

Rheumatoid arthritis (RA) is an inflammatory condition characterized by systemic polyarthritis with bone erosion that affects the peripheral joints. The etiology of RA remains unclear with immunological processes including T cell-B cell interactions, innate immunity, and cytokine activity being implicated (1). In RA, the synovium contains many infiltrating mononuclear cells including B cells at various developmental stages, T cells, and macrophages (2).

Treatments for RA include agents that target cytokines such as tumor necrosis factor. These inflammation-neutralizing approaches have achieved good results in reducing not only joint inflammation but also bone erosion. More recently, depletion of B cells from RA patients has also produced significant therapeutic benefits in several clinical trials (3,4). B cells are thought to be crucial in the pathogenesis of RA, through the production of autoantibodies, antigen presentation, cytokine secretion, and costimulatory signaling. In fact, autoantibodies including rheumatoid factor and anti-cyclic citrullinated peptide antibody (Ab) have been used as diagnostic markers of RA. However, most such autoantibodies are not pathogenic. In contrast, anti-glucose-6-phosphate isomerase (GPI) Ab is a candidate arthritogenic Ab, identified using the K/BxN arthritis model (5-7). In this model, disease development was initiated by the activation of B and T cells. In addition, B cell-deficient mice do not develop arthritis (8). Anti-GPI Ab is also detected in some RA patients, with the reported prevalence varying from 5% to 64% of RA patients (9-12). This Ab is associated with extra-articular manifestations, and its titer correlates with the disease activity (10,13). Moreover, anti-GPI Ab is also detected in the inflamed synovium of RA (14-16). Thus, B cells and autoantibodies appear to play important roles in the pathogenesis of RA in anti-GPI Ab-positive patients, especially in the inflamed joint synovium.

Immunoglobulin molecules are composed of two heavy chains and two light chains, and are characterized by the

Table I. Profile of participating patients (RA1-RA6).

	Age (years)	Sex	Disease duration (years)	Anti-GPI Ab (OD 405 nm)		RF (IU/ml)	CRP (mg/dl)	MMP-3 (ng/ml)
				Human	Rabbit			
RA1	66	F	20	1.78	3.14	156	0.57	363
RA2	70	F	25	2.60	3.47	149	0.42	275
RA3	69	F	16	2.43	2.55	516	3.55	295
RA4	64	F	33	0.72	0.20	78	0.05	ND
RA5	72	F	22	0.62	0.05	119	0.74	215
RA6	74	F	20	0.40	0.36	5	0.56	256

All synovia were from the knees of female patients with rheumatoid arthritis (RA). The cutoff OD was calculated from ELISA reaction of 145 healthy Japanese donors, the mean value + two standard deviation was 1.32 to human recombinant GPI, and 0.94 to rabbit native GPI. Double-positive populations were considered anti-GPI Ab-positive. RA1-3 were anti-glucose-6-phosphate isomerase (GPI) Ab (+), and RA4-6 were anti-GPI Ab (-). Apart from anti-GPI Ab, all other parameters were matched to the utmost extent. GPI, glucose-6-phosphate isomerase; RF, rheumatic factor; CRP, C-reactive protein; MMP-3, matrix metalloproteinase-3; ND, not done.

antigen-binding site sequence and Fc isotype. The antigen-binding site is made up of variable regions and rearranged complementarity-determining regions (CDR) that determine the individual immune properties of any given B cell. The immunoglobulin heavy chain CDR3 (IgH-CDR3) is the most crucial site for antigen binding. H-CDR3 is rearranged by one of 44 variable segments (VH), one or more of 25 diversity segments (DH), and one of six joining segments (JH) (17). In addition, VH genes can be divided into seven sub-families (VH1 to VH7), with overrepresentation of VH4 genes reported in some autoimmune conditions (18). Negative selection of VH4 repertoires is implemented in healthy individuals to avoid autoimmunity (19,20). On the other hand, in RA patients, synovial B cells, especially plasma cells, are also biased to express the VH4 repertoire (21,22), though this was negated in another report (23), and antigen-driven affinity maturation has been reported (23-26).

The present study defined the synovial B cell characteristics of anti-GPI Ab-positive (+) RA patients by analyzing VH regions of synovial B cells from anti-GPI Ab (+) and negative (-) RA patients and compared the rearranged IgH-CDR3 sequences of their VH4 genes. Twenty-seven IgH-VH4 gene sequences from anti-GPI Ab (+) RA patients were compared with thirty-six VH4 gene sequences from anti-GPI Ab (-) patients. In both groups, over 70% of VH4 clones seemed to be undergoing antigen-driven maturation, as evidenced by an R/S ratio of >3 in the CDR and less in the framework region (FR). However, the JH4 gene was more predominant in the synovium of anti-GPI Ab (+) RA patients compared to anti-GPI Ab (-) cases and the lengths of their IgH-CDRs were shorter. In addition, there was no biased usage of VH4 subfamily genes. Together, these findings suggest that B cells from anti-GPI Ab (+) RA synovium are affinity-matured by antigens, with frequent usage of VH4-JH4.

Materials and methods

Subjects. Synovial tissues were obtained from six patients [three were anti-GPI Ab (+) and three were anti-GPI Ab (-)] who satisfied the American College of Rheumatology criteria for RA (1987) (27) (Table I). For selecting anti-GPI Ab-positive patients, enzyme linked immunosorbent assay (ELISA) was performed using two different sources of GPI; a recombinant human GPI (huGPI), and a rabbit muscle GPI (raGPI) (Sigma Chemical Co., St. Louis, MO) which had been described in detail previously (12). Informed consent for using synovial tissues and blood sampling was obtained from all patients at the time of the relevant procedure.

Table II. Specific primers for each VH family.

Internal	
VH1	5'-TCACCATGGACTGCACCTGGA-3'
VH2	5'-CCATGGACACACTTTGCTCCAC-3'
VH3	5'-TCACCATGGAGTTTGGGCTGAGC-3'
VH4	5'-AGAACATGAAACACCTGTGGTTCTT-3'
VH5	5'-ATGGGGTCAACCGCCATCCT-3'
VH6	5'-ACAATGTCTGTCTCCTTCCTCAT-3'
Cγ	5'-CATCGGTCTTCCCCCTGGC-3'
External	
VH1	5'-GAGAAAACCCTGTGAGCACAGCT-3'
VH2	5'-AGTGACTCCTGTGCCCCAC-3'
VH3	5'-GATCAGCACTGAACACAGAGGAC-3'
VH4	5'-GTCATGGACCTCCTGCACAAG-3'
VH5	5'-AGGGCTTCATTTTCTGTCCTCCAC-3'
VH6	5'-GGGGCAGTCACCAGAGCTC-3'
Cγ	5'-GAGCACCTCCGAGAGCACA-3'

Sequences of primers used in nested PCR to detect immunoglobulin heavy chain variable segment (VH) 1-6 family genes.

cDNA synthesis. Synovial tissues were minced and homogenized in Isogen (Nippon Gene, Tokyo, Japan) and extracted with chloroform. RNA was precipitated with isopropanol,

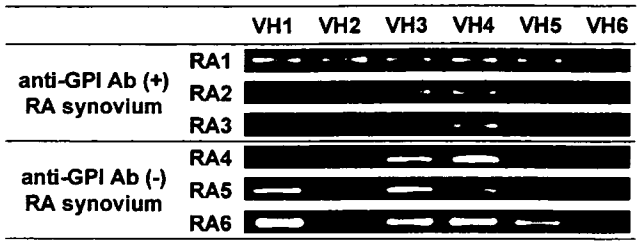


Figure 1. Nested PCR for immunoglobulin heavy chain variable segment (VH) subfamily of rheumatoid synovium. Amplification of VH genes by nested PCR was conducted as shown. Only VH4 genes were amplified in all individuals. VH1 to VH5 were amplified in all cases except RA3.

resuspended in 10 mM Tris·HCl (pH 8.0) and 1 mM EDTA. RevertAid first-strand cDNA synthesis kits (Fermentas, Ontario, Canada) were used for reverse transcription, in accordance with the manufacturer's instructions. Total RNA (5 μg) was used for this reaction.

Amplification of VH genes. Rearranged immunoglobulin VH genes were amplified by PCR. Nested PCR was conducted to obtain sufficient PCR product for electrophoresis. To avoid sequence errors, the first PCR products were subjected to sequencing. Specific oligonucleotides for six different VH families (VH1 to VH6) were used as 5'-primers. Oligonucleotide corresponding to the known Cγ was used as a 3'-primer. For the second PCR, an additional set of primers using the internal sequences was prepared (Table II). One microliter of template was added to 24 μl of a PCR master mix, containing 1.25 U rTaq DNA polymerase (Takara Bio Inc, Shiga, Japan), 2.5 μl of manufacturer's 10X PCR buffer, 2 μl of 2.5 mM dNTPs, and 2.5 μl of each 10-μM primer. The first round of PCR was performed for 25 cycles at 95°C for 30 sec to denature, 54 or 56°C for 30 sec to anneal (annealing for VH1, VH2, VH3, VH5 at 56°C, and for VH4, VH6 at 54°C), 72°C for 30 sec to elongate including a previous 5 min of heating at 95°C to unfold the cDNA, and a final 7-min extension at 72°C. The second amplification was performed using 1 μl of the first PCR products as templates and the same method as for the first amplification.

Sequence analysis. Aliquots of the first PCR products were separated by electrophoresis using a 1.5% agarose gel, and DNA bands in the range of 400 bp were purified from the agarose gel using a MinElute gel extraction kit (Qiagen, Hilden, Germany). Purified PCR products were cloned into the TA cloning vector (Invitrogen, San Diego, CA). Randomly picked clones were screened for inserts of 400 bp. Positive clones were subjected to cycle sequencing using a BigDye terminator cycle sequence kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The sequences were determined by capillary sequencer (Applied Biosystems 310 genetic analyzer). BioEdit (Ibis Therapeutics, Carlsbad, NM) was used for sequence comparison, and JoinSolver [National Institutes of Health, National Institute of Arthritis and Musculosketelal and Skin Diseases (NIAMS) and the Center for Information Technology (CIT)] (28) was used to identify putative Ig-VDJ germline sequences and to clarify the ratio of mutations leading to amino acid replacement to silent mutations (R/S ratio) in the CDR and FR. The IMGT database (Marie-Paule Lefranc, Montpellier, France) was used to confirm the putative Ig-VDJ germline sequences. EMBL Nucleotide Sequence Submissions (European Bioinformatics Institute, Wellcome Trust Genome Campus, Cambridge, UK), and GenBank (National Institutes of Health, Bethesda, Maryland) were also used.

Statistical analysis. The Mann-Whitney U test was used to compare the IgH-CDR3 length of VH between anti-GPI Ab (+) and (-) patients. The two-tailed Fisher exact test was used to determine significant differences in distributions of JH gene usage. Data are expressed as mean ± SD. A p value <0.05 was considered statistically significant.

Results

Amplification of VH family genes. Only VH4 genes were identified from all patients by nested PCR (Fig. 1). VH1 to VH5 genes were also identified except for RA3. In contrast, the amplification of VH6 genes was not sufficient for detection.

R/S ratio. After the first PCR, the VH4 gene products were purified and sequenced. Twenty-seven (17, 8, and 2, respectively) individually rearranged VH4 genes were characterized by sequence analysis in the synovium of anti-GPI Ab (+) RA patients (Table III), and 36 (15, 19, and 2, respectively) were identified in the synovium of anti-GPI Ab (-) individuals (Table III). There was no difference in R/S ratio in the CDR of both groups of VH4 clones [70% (19/27) in anti-GPI Ab (+) and 75% (27/36) in anti-GPI Ab (-)]; the R/S ratio in the CDR was >3, indicating antigen-driven maturation.

VH4 subfamily. Sequences were analyzed by using JoinSolver software to determine the implicated VDJ usage. In the synovium of anti-GPI Ab (+) RA patients, the most frequent VH4 subfamily gene was VH4-59 (12 products) followed by VH4-4 (5 products), VH4-39 (4 products), VH4-31 (3 products), VH4-61 (2 products), and finally, VH4-34 (1 product). In anti-GPI Ab (-) individuals, the dominant detected VH4 subfamily gene was VH4-59 (12 products) followed by VH4-39 (10 products), VH4-31 and VH4-61 (5 products each), and VH4-4 and VH4-34 (2 products each) (Fig. 2a). VH4-39 was therefore relatively less frequent in synovial B cells of anti-GPI Ab (+) RA patients, although the statistical significance was not clear.

JH region. In the synovium of anti-GPI Ab (+) RA patients, the most frequent JH gene was JH4 (19 products) followed by JH3 (3 products), JH6 (2 products), and JH1, JH2, and JH5 (1 product each). In anti-GPI Ab (-) individuals, the most frequent synovial JH gene was JH5 (10 products) followed by JH6 (9 products), JH3 and JH4 (7 products each), and JH2 (3 products) (Fig. 2b). Thus, JH4 usage in synovial B cells of anti-GPI Ab (+) RA patients was 70%, and showed a significantly higher frequency compared to 19% usage in anti-GPI Ab (-) individuals ($p<0.0001$).

IgH-CDR3 characteristics: amino acid composition and IgH-CDR3 length. There was no statistically significant difference

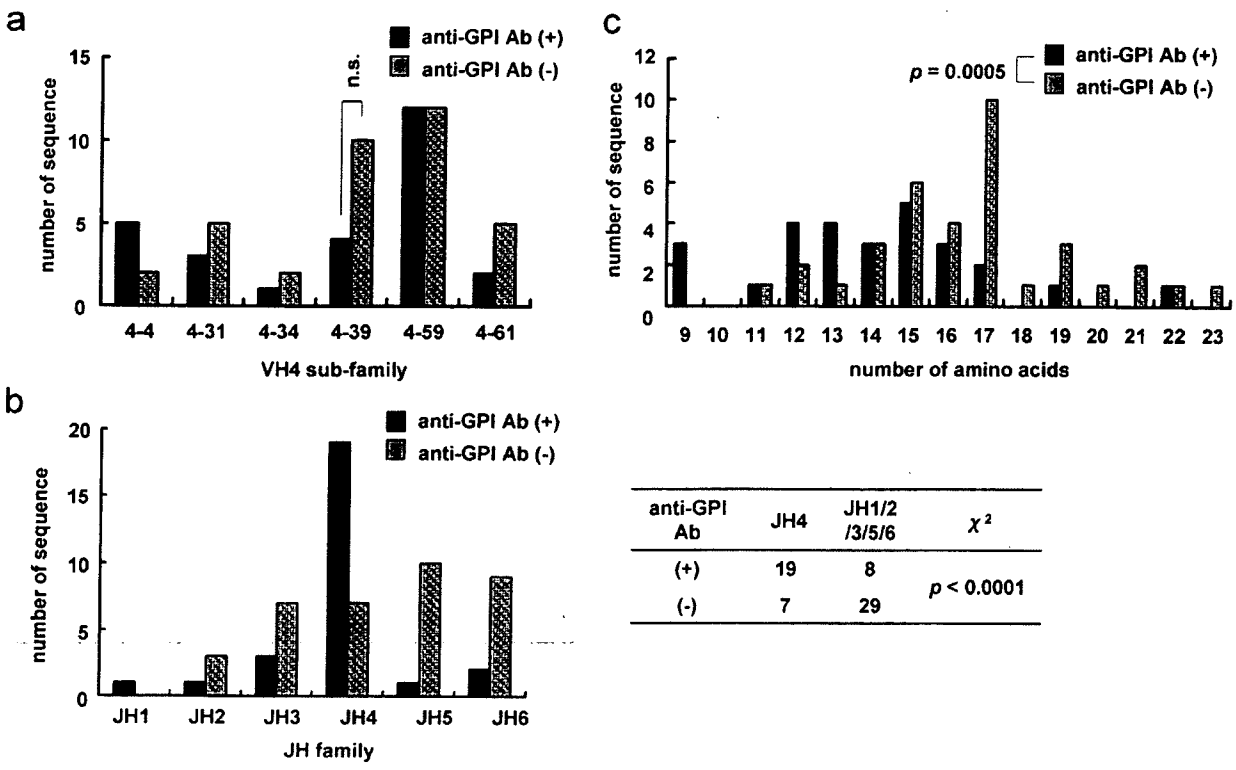
Table III. Synovial immunoglobulin VH4 repertoire of anti-GPI Ab (+) and (-) RA patients.

	VH	DH	H-CDR3	bp	JH	R/S ratio within CDR
Anti-GPI Ab (+) RA patients						
RA1	4-04	2-21	NMAGDVIGFFDY	14	4	2/0
	4-04	4-23	**S**R**R**NPIDYPLGYFDY	16	4	2/1
	4-04	5-05	GYSYGLFDV	11	4	12/2
	4-04	6-25	Q**RHRR**GDFDI	12	3	2/0
	4-31	3-10	EEL**RRIR**GPFFDY	15	4	9/3
	4-34	6-06R	GEQDE**H**QVSS**R**FFFYYYIDV	22	6	9/2
	4-39	2-15	QGYCSGGTCQDFDY	16	4	3/0
	4-39	3-10	QGA**R**QWFGEFGAFDY	17	4	6/0
	4-59	3-09	LSPGGNFDFDL	13	4	6/1
	4-59	3-10	DGEGGSYYFDY	13	4	9/1
	4-59	3-10R	**H**NNTW**H**PFDY	12	4	8/1
	4-59	3-16R	LPP**R**GNY**R**LDS	13	4	5/1
	4-59	6-13	VPGFSSTWFEVDY	15	4	6/2
	4-59	6-13	FSGSFYGWFDP	13	5	7/0
	4-59	IR	VSTQTDY	9	4	5/1
	4-59	1-07	APPPWL**RR**VSTGTWL	17	2	5/3
	4-61	2-02R	GRQPDYYYAMDV	14	6	8/4
RA2	4-04	5-12R	SPDN**R**NTLDI	12	3	7/2
	4-31	3-10	GYYYGPGSY**H**PFET	16	4	3/1
	4-31	6-13	D**R**DAAAG**R**WVDY	14	4	3/0
	4-39	1-26	PVVGA**R**DPAPFDL	15	3	4/2
	4-59	3-03	**R**GGPTE**H**	9	1	4/1
	4-59	3-09	D**R**GQEYGIDS	12	4	4/2
	4-59	IR1R	LGQLGD**H**	9	4	12/2
	4-61	1-20	VSLLGY**KR**NDG**K**Y**H**FDY	19	4	6/2
RA3	4-39	3-10	YI**R**GV**R**SGGYFDY	15	4	4/2
	4-59	1-26	**H**GVDSGSFYAFDY	15	4	3/0
Anti-GPI Ab (-) RA patients						
RA4	4-31	3-10	D**H**GSGSSYFFSPNYGMDV	20	6	3/1
	4-34	5-12	GNSGNGYYFYNYMDV	17	6	11/3
	4-39	2/OR15-2R	FTITLF**RGK**EGNY	15	4	5/0
	4-39	3/OR15-3	QNGLQS**R**VDYFDF	15	4	7/1
	4-39	IRR	GGGVNLGSGAFYDE	16	4	18/7
	4-59	1-01	GGGFSSNWSLAPFAFDI	19	3	3/2
	4-59	2-15	DVDCVGGSCYSSDWFDP	19	5	5/1
	4-59	3-22	LWGSSGLYGENWFDP	17	5	5/1
	4-59	4/OR15-4	DVTSVQTTMVPAFDY	17	4	9/2
	4-59	5-05	DI**R**GYGYGYFDL	14	2	13/1
	4-59	6-19	DT**H**TAVPGDDYFES	16	4	7/3
	4-61	1-26	ESL**K**VGSTCFDP	14	5	9/3
	4-61	3-10	A**R**PDGSESFY**R**YLDL	17	2	4/1
	4-61	3-10	EQTGL**R**GQNM	12	3	7/2
	4-61	4-23	EGDYGGSYYYYYMDL	17	6	11/0
RA5	4-04	2-15	AGGGDCSGATCYSYYYGMDV	22	6	5/0
	4-31	2-21	GFGSSVIAMAYYFDY	17	4	3/1
	4-31	4-04	L**H**AE**R**ALGFWFDP	15	5	17/3
	4-31	4/OR15-4	VAPGAMPDDASEI	15	3	8/1
	4-34	3-09	MANLTGTPGLGI	14	3	7/2
	4-39	2/OR15-2R	DYITIFGVAPFDP	15	5	4/1

Table III. Continued.

	VH	DH	H-CDR3	bp	JH	R/S ratio within CDR
	4-39	3-03	**H**VNFEVVIGRWFD**H**	16	5	13/3
	V39	3-03	LGALFGADSYYGMDV	17	6	6/1
	V39	4-23	**K**DYADYEGFAY	13	5	6/0
	V39	5-12	YISATMEDF	11	3	11/2
	V39	6-13	DAGYSSS**RH**PVGFDP	17	5	8/3
	V39	6-19R/3-16	**HAR**IGA**H**YTYGSF**R**LFDAFDV	23	3	5/1
	V59	3-03	D**K**SGYYTPGGYYYYYGMDV	21	6	3/2
	V59	3-03	APYWSGYVYGLDV	15	6	7/1
	V59	3-10	ETYYSASGSYYSGQYYFEY	21	4	6/1
	V59	4/OR15-4	**H**GGLYPYYYFAMDV	16	6	5/0
	V59	6-19	**R**TDDYS**R**GWYWYFDP	17	2	6/1
	V59	6-19R	**HAIHR**FSTAFPNWFDP	18	5	3/2
	V61	4-17	DASLLYGDYVSWFDP	17	5	8/5
RA6	V04	1-14R	DP**R**TV**K**TMDV	12	6	6/4
	V59	3-22	GP**H**DTMTNYYGLNAFDI	19	3	7/4

The characters of immunoglobulin sequences using VH4 family genes are shown. Bold characters represent based-ionized amino acids (R, arginine; H, histidine; K, lysine). Underline indicates R/S ratio >3.



anti-GPI Ab	JH4	JH1/2 /3/5/6	χ^2
(+)	19	8	$p < 0.0001$
(-)	7	29	

Figure 2. Comparison of synovial VH4 B cells from rheumatoid arthritis (RA) patients with or without anti-glucose-6-phosphate isomerase (GPI) Ab. (a) Usage of VH4 subfamily frequencies of VH4 gene usage in synovial B cells from RA patients are shown. In anti-GPI Ab (+) patients, VH4-59 (12 products), VH4-4 (5 products), VH4-39 (4 products), VH4-31 (3 products), VH4-61 (2 products), and VH4-34 (1 products) were identified. In anti-GPI Ab (-) patients, VH4-59 (12 products), VH4-39 (10 products), VH4-31 and VH4-61 (5 products each), and VH4-4 and VH4-34 (2 products each) were identified. VH4-39 showed a relatively low frequency in synovial B cells of anti-GPI Ab (+) RA patients, although the comparison is statistically not significant. (b) The usage of immunoglobulin heavy chain joining segment (JH) family. The frequencies of JH gene usage of synovial VH4 B cells from RA patients are shown. JH4 (19 products) was the most frequent gene used in anti-GPI Ab (+) RA patients, although this gene was not predominant in anti-GPI Ab (-) individuals ($p<0.0001$ by two-tailed Fisher exact test between JH4 and others). (c) The number of amino acids in the immunoglobulin heavy chain complementarity-determining region 3 (IgH-CDR3). The IgH-CDR3 lengths of VH4 B cells are shown. Lengths varied between 9 and 23 (mean, 15.46 ± 3.09) amino acids. In anti-GPI Ab (+) RA patients, the IgH-CDR3 lengths (14.00±2.96 amino acids) of synovial VH4 B cells were significantly shorter than those of anti-GPI Ab (-) individuals (16.56±2.75 amino acids) ($p=0.0005$ by Mann-Whitney's U test).

between the groups in terms of amino acids usage of IgH-CDR3, although this region in anti-GPI Ab (+) RA patients was rich in basic-ionized amino acids (arginine, histidine, and lysine) in their near central position, compared to the composition in anti-GPI Ab (-) individuals (Table III).

The IgH-CDR3 amino acid lengths varied from 9 to 23 amino acids (mean, 15.46±3.09 amino acids). In anti-GPI Ab (+) RA patients, the IgH-CDR3 length of synovial immunoglobulins using VH4 was significantly shorter than the length of those in anti-GPI Ab (-) individuals (p=0.0005, Fig. 2c). These findings suggest the prevalence of affinity-matured VH4 B cells in the synovium of anti-GPI Ab (+) RA patients.

Discussion

Anti-GPI Ab is frequently detected in patients with aggressive forms of RA (12,13), and its level correlates significantly with extra-articular manifestations such as rheumatoid nodules, rheumatoid vasculitis, and Felty's syndrome (10). We reported previously that serum IgG from anti-GPI Ab (+) RA patients preferentially attached to the articular surface of the meta-carpophalangeal joints of the monkey, inducing recruitment of granulocytes and mononuclear cells into the synovium (29). These results indicated that human serum immunoglobulins from RA patients include autoantibodies to specific protein(s) expressed in the joint cavity. Furthermore, human GPI protein is expressed on the cartilage and synovial surface in RA (7) and anti-GPI Ab is present in the synovial fluid, suggesting that the local production of such autoantibodies might be associated with arthritis. To address this hypothesis, we focused on the synovial B cells of anti-GPI Ab (+) patients.

In the present study, VH4 genes were detected in the synovium of all patients with RA. In some autoimmune diseases such as systemic lupus erythematosus (SLE) (30,31), VH4 genes are overrepresented in peripheral B cells (18), although negative selection of VH4 genes occurs in healthy individuals (19,20). These observations implicate VH4 genes as a self-reactive gene family. The frequency of VH4 genes in peripheral B cells from RA patients was not different from that of healthy individuals (32), however VH4 genes were highly expressed in the rheumatoid synovial B cells (21,22). In addition, antigen-driven immune maturation of B cells is characterized by an R/S ratio >3 within the CDR (33). Our study demonstrated that VH4 (+) synovial B cells in patients with RA are affinity-matured, because immunoglobulins with a high R/S ratio were dominant.

A skewed VH4 subfamily in RA synovium was not identified in this study, but when we compared anti-GPI Ab (+) with (-) patients, VH4 subfamily usages of synovial B cells from anti-GPI Ab (+) RA patients were less frequent for VH4-39. In peripheral blood of SLE, VH4-34 (V4.21) was overexpressed and correlated with some autoantibodies (30,31), but no specific subfamily repertoire has been identified in the synovium of RA patients. We do not know whether these skewed VH4 subfamilies are related to arthritogenicity, however, there are reports that some autoantibodies with VH4-34 (V4.21) segments are related to the pathogenicity of SLE (34,35). Since anti-GPI Ab is a candidate arthritogenic antibody, it would be interesting to identify the skewed VH4 subfamilies by increasing numbers of the sequence.

Our sequence analysis noted that synovial IgH-CDR3 from anti-GPI Ab (+) RA patients was enriched in basic-ionized amino acids. The ionized side-chains of arginine in the CDRs contribute to higher binding affinity for some antigens such as DNA, cardiolipin (36,37), and TAG72 (38). A previous study found that arginine in IgH-CDR3 of human and murine anti-dsDNA was most likely to be generated during V-D-J rearrangement in B cells, and the higher frequency of arginine in the IgH-CDR might similarly be due to the clonal expansion of B cells (38). In addition, the precise location of arginine is important for the binding (37).

IgH-CDR3 length and amino acid composition is the major contributor to antigen specificity and affinity (39-41). Matured immunoglobulins have shorter CDR3s than non-matured ones in both mice and humans (42,43). In the present anti-GPI Ab (+) RA patients, the CDR3 length of synovial immunoglobulins using VH4 was significantly shorter and the JH4 usage was significantly higher than those of anti-GPI Ab (-) individuals. These data support the notion that synovial B cells of anti-GPI Ab (+) patients are affinity-matured with higher affinity to a particular antigen.

In conclusion, our findings on synovial B cells in RA patients positive for anti-GPI Ab clearly demonstrated a high frequency of VH4-JH4 subfamily genes rich in basic amino acids and shorter CDR3 length, indicating affinity-matured B cells, reactive to autoantigens such as GPI. Future studies using anti-GPI Ab-producing B cell hybridomas should shed light on the functional role of anti-GPI Ab in the pathogenesis of RA.

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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

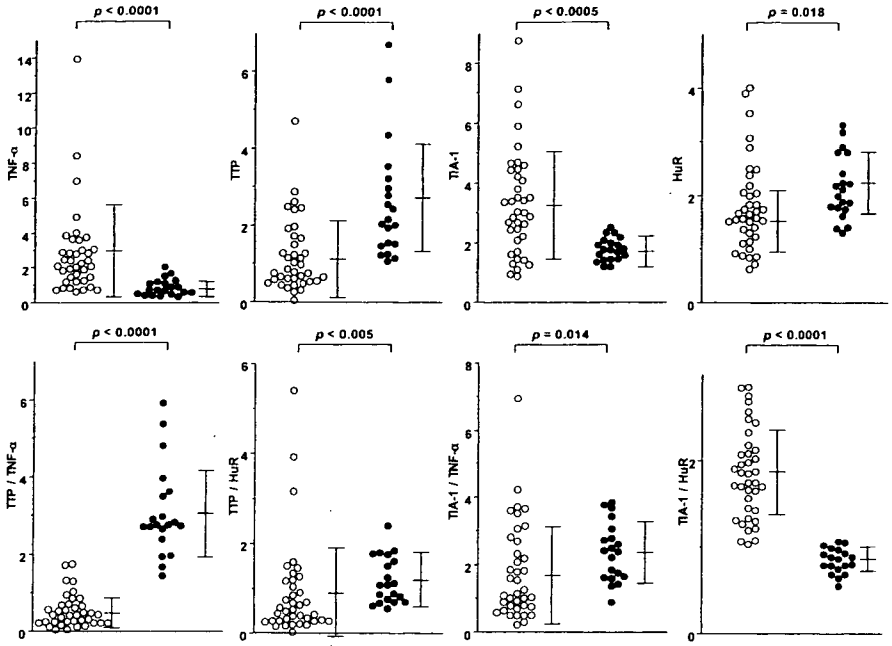

Figure 1. Expression levels of the tumor necrosis factor α (TNFα) gene and 3 adenine/uridine-rich element binding protein (tristetraprolin [TTP], T cell intracellular antigen 1 [TIA-1], and Hu antigen R [HuR]) genes in peripheral blood mononuclear cells from 38 patients with active rheumatoid arthritis at week 0 (open circles) and from 20 healthy control subjects (solid circles). Bars show the mean ± SD. *P* values were calculated by Mann-Whitney U test.

and 2.84 ± 1.99 at week 2; for TTP, 1.20 ± 0.95 at week 0 and 1.17 ± 1.32 at week 2; for TIA-1, 3.34 ± 1.79 at week 0 and 3.88 ± 1.79 at week 2; for HuR, 1.79 ± 0.83 at week 0 and 2.06 ± 0.91 at week 2) (Figure 2). However, the TTP:HuR gene expression ratio decreased 2 weeks after initiation of infliximab therapy (0.90 ± 1.09 at week 0 and 0.71 ± 0.88 at week 2; $P = 0.015$), while no significant changes were noted in the TTP:TNFα, TIA-1:TNFα, and TIA-1:HuR ratios (for TTP:TNFα, 0.55 ± 0.43 at week 0 and 0.50 ± 0.39 at week 2; for TIA-1:TNFα, 1.80 ± 1.42 at week 0 and 1.94 ± 1.19 at week 2; for TIA-1:HuR, 1.85 ± 0.52 at week 0 and 1.88 ± 0.34 at week 2) (Figure 2).

At week 54, the TNFα gene expression level and the TIA-1:HuR gene expression ratio increased from those observed at week 0 (for TNFα, 2.80 ± 2.48 at week 0 and 5.05 ± 3.89 at week 54 [$P = 0.015$]; for TIA-1:HuR, 1.85 ± 0.52 at week 0 and 2.35 ± 0.71 at week 54 [$P = 0.010$]). TTP gene expression increased from that at week 2 (1.17 ± 1.32 at week 2 and 1.61 ± 0.94 at week 54; $P = 0.0065$). In contrast, the TIA-1:TNFα gene expression ratio decreased, from 1.94 ± 1.19 at week 2 to 1.22 ± 0.81 at week 54 ($P = 0.026$). Fluctuations in TIA-1 gene expression and the TTP:TNFα and TTP:HuR gene expression ratios differed greatly among individual patients (Figure 2).

Relationship between TNFα and ABP gene expression levels in patients with RA. We next examined the correlation between the gene expression levels in PBMC samples from healthy control subjects and those in samples from patients with RA at week 0, week 2, and week 54. We anticipated that although posttranscriptional regulation of TNFα production would be adequately executed in healthy individuals, some disturbance might be present in patients with active RA. These disturbances may be partially responsible for the higher disease activity in these patients to whom infliximab is prescribed. In particular, we were interested in investigating whether the correlation between TNFα and the ABPs that have been shown to suppress TNFα production (TTP and TIA-1) would be altered. In addition, we were interested in determining whether a disturbance in posttranscriptional regulation of TNFα production, if it does exist, would be affected by infliximab therapy.

In the control samples, gene expression of TNFα correlated with the expression levels of all genes examined (for TTP and TNFα, r = 0.64 and $P = 0.0017$; for

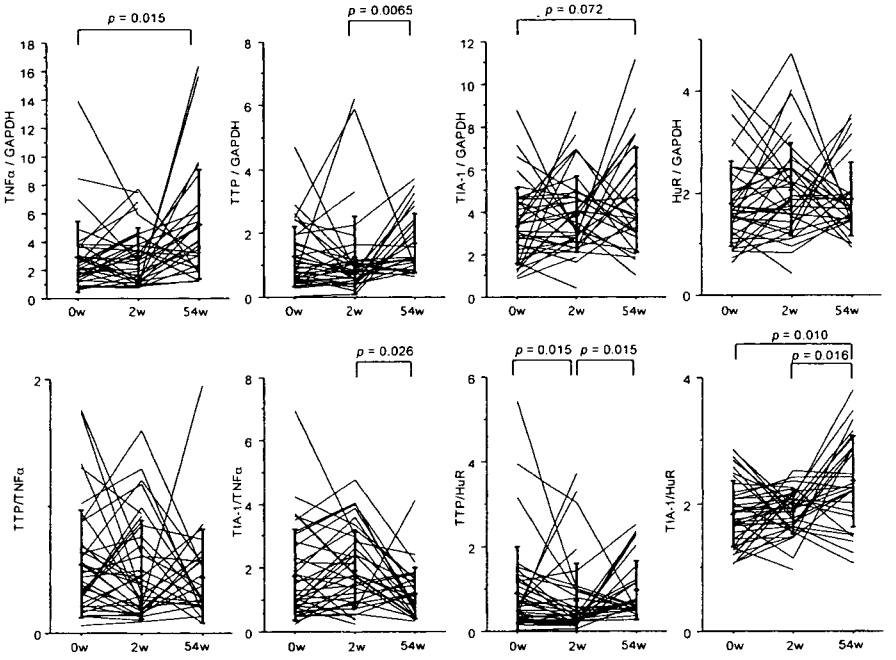

Figure 2. TNFα and adenine/uridine-rich element binding protein gene expression levels in peripheral blood mononuclear cells from patients with rheumatoid arthritis, before (0w) and 2 weeks and 54 weeks after administration of the first dose of infliximab. Bars show the mean ± SD. *P* values were calculated by Wilcoxon's rank sum test. See Figure 1 for definitions.

TTP and HuR, r = 0.64 and $P = 0.0017$; for TIA-1 and TNFα, r = 0.62 and $P = 0.0030$; for TIA-1 and HuR, r = 0.73 and $P = 0.0001$; for TNFα and HuR, r = 0.60 and $P = 0.0041$; for TTP and TIA-1, r = 0.59 and $P = 0.0049$) (Figure 3A). In samples obtained from patients with RA at week 0, the correlations between TTP and TNFα and between TIA-1 and HuR were significant (for TTP and TNFα, r = 0.40 and $P = 0.016$; for TIA-1 and HuR, r = 0.87 and $P < 0.0001$) (Figure 3B), while the correlation between TTP and HuR was not significant. Similar correlations were noted at week 2 and at week 54 (for TTP and TNFα, r = 0.34 and $P = 0.039$ at week 2 and r = 0.39 and $P = 0.042$ at week 54; for TIA-1 and HuR, r = 0.94 and $P < 0.0001$ at week 2 and r = 0.84 and $P < 0.0001$ at week 54) (Figures 3C and D).

Interestingly, the significant relationships for gene expression between TIA-1 and TNFα, TNFα and HuR, and TTP and TIA-1 were not observed in samples obtained from patients with RA at week 0. However, significant relationships between TIA-1 and TNFα and between TNFα and HuR gene expression were observed in week 2 and week 54 samples (for TIA-1 and TNFα, r = 0.18 and $P = 0.27$ at week 0, r = 0.38 and $P = 0.022$ at week 2, and r = 0.51 and $P = 0.009$ at week 54; for TNFα and HuR, r = 0.10 and $P = 0.54$ at week 0, r = 0.45 and $P = 0.007$ at week 2, and r = 0.72 and $P = 0.0002$ at week 54 (Figures 3B, C, and D). These observations suggest that regulatory mechanisms that control the expression of these molecules are disturbed in patients with active RA and are somewhat restored after the initiation of anti-TNFα therapy.

Relationship between TNFα and ABP gene expression and efficacy of infliximab therapy. Our working hypothesis was that differences in the regulation of ABP production might lead to differences in the severity of RA and the efficacy of TNFα-blocking agents. Thus, we anticipated that we might observe some differences in the expression of these molecules between patients whose disease responded to infliximab and infliximab nonresponders.

At the time of this study, 27 patients with RA had received at least 9 courses of infliximab therapy (week 54). At week 54, 18 patients (66.7%) had achieved at least an ACR20 response; 14 patients (51.9%) had achieved an ACR50 response, and 8 patients (29.6%) had achieved an ACR70 response. The 14 patients who achieved an ACR50 response at week 54 were included in the responder group, while the 9 patients who did not