Table 5

Correlation between anti-mouse glucose-6-phosphate isomerase (GPI) antibodies titres and arthritis score

Immunisation	Rho value	P value
rhGPI	-0.825	0.0989
h325–339	-0.525	0.2937
h325–339 plus h544–558	0.500	0.3173

Sera were taken on day 14 from mice immunised with recombinant human (rh) GPI, hGPI$_{325-339}$ or hGPI$_{325-339}$ plus hGPI$_{544-558}$. The correlation between the titres of anti-GPI antibodies and arthritis score on day 14 were statistically analysed with the Spearman's rank correlation coefficient. In the case of five samples, Rho values above 0.900 indicate significant positive correlation between anti-mouse GPI antibody titres and arthritis score, whereas Rho values below - 0.900 indicate significant negative correlation (p < 0.05). Five mice per group. Representative data of two independent experiments.

administration of a dominant epitope leads to anergy of pathogenic T cells or results in activation-induced cell death [30,31], this inhibitory effect of hGPI$_{325-339}$ on GPI-induced arthritis supports the notion that hGPI$_{325-339}$ may be the dominant epitope.

Cross-reactivity is considered the one of mechanisms of autoimmune diseases. We previously identified patients with RA who have GPI-reactive CD4+ T cells and found that some of them express human leucocyte antigen-DR4 as MHC class II [32]. Because the I-A^q binding motif resembles DR4 [9], further studies are needed to define epitopes of CD4+ T cells in such patients and search proteins that have homology to the epitopes.

Conclusions

This study is the first report of experimental arthritis induced by immunisation with a single short peptide in genetically unaltered mice. The fact that an immunological reaction to a single short peptide of ubiquitously expressed protein causes polyarthritis provides new insight to the understanding of autoimmune arthritis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KI wrote the manuscript and conceived of the study. YT and AI assisted experiments and statistical analysis. IM and TS participated in its full design and coordination, and DG, SI and AK participated in discussions.

Acknowledgements

References

1. Schubert D, Maier B, Morawietz L, Krenn V, Kamradt T: Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in generally unaltered mice. J Immunol 2004, 172:4503-4509.
2. Iwanami K, Matsumoto I, Tanaka-Watanabe Y, Mihira M, Ohsugi Y, Mamura M, Goto D, Ito S, Tsutsumi A, Kishimoto T, Sumida T: Crucial role of IL-6/IL-17 axis in the induction of arthritis by glucose-6-phosphate isomerase. Arthritis Rheum 2008, 58:754-763.
3. Matsumoto I, Zhang H, Yasukochi T, Iwanami K, Tanaka Y, Inoue A, Goto D, Ito S, Tsutsumi A, Sumida T: Therapeutic effects of antibodies to tumor necrosis factor-α, interleukin-6 and cytotoxic T-lymphocyte antigen 4 immunoglobulin in mice with glucose-6-phosphate isomerase induced arthritis. Arthritis Res Ther 2008, 10:R66.
4. Brand DD, Myers LK, Terato K, Whittington KB, Stuart JM, Kang AH, Rosloniec EF: Characterization of the T cell determinants in the induction of autoimmune arthritis by bovine α1(II)-CB11 in H-2^q mice. J Immunol 1994, 152:3088-3097.
5. Matsumoto I, Lee DM, Goldbach-Mansky R, Sumida T, Hitchon CA, Schur PH, Anderson RJ, Coblyn JS, Weinblatt ME, Brenner M, Duclos B, Pasquali JL, El-Gabalawy H, Mathis D, Benoist C: Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and a spectrum of other chronic autoimmune disorders. Arthritis Rheum 2003, 48:944-954.
6. Matsumoto I, Staub A, Benoist C, Mathis D: Arthritis provoked by linked T and B recognition of a glycolytic enzyme. Science 1999, 286:1732-1735.
7. MHCPred [http://www.jenner.ac.uk/MHCPred/]
8. Kawamoto T: Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. Arch Histol Cytol 2003, 66:123-143.
9. Bayrak Ş, Holmdahl R, Travers P, Lauster R, Hesse M, Dölling R, Mitchison NA: T cell response of I-A^q mice to self type II collagen: meshing of the binding motif of the I-A^q molecule with repetitive sequences results in autoreactivity to multiple epitopes. Int Immunol 1997, 9:1687-1699.
10. Chen JS, Lorenz RG, Goldberg J, Allen PM: Identification and characterization of a T cell-inducing epitope of bovine ribonuclease that can be restricted by multiple class II molecules. J Immunol 1991, 147:3672-3678.
11. Fritz RB, Skeen MJ, Chou CH, Garcia M, Egorov IK: Major histocompatibility complex-linked control of the murine immune response to myelin basic protein. J Immunol 1985, 134:2328-2332.
12. Sakai K, Sinha AA, Mitchell DJ, Zamvil SS, Rothbard JB, McDevitt HO, Steinmann L: Involvement of distinct murine T-cell receptors in the autoimmune encephalitogenic response to nested epitopes of myelin basic protein. Proc Natl Acad Sci USA 1988, 85:8608-8612.
13. Myers LK, Cooper SW, Terato K, Seyer JM, Stuart JM, Kang AH: Identification and characterization of a tolerogenic T cell determinant within residues 181-209 of chick type II collagen. Clin Immunol Immunopathol 1995, 75:33-38.
14. Michaëlsson E, Andersson M, Engström A, Holmdahl R: Identification of an immunodominant type-II collagen peptide recognized by T cells in H-2^q mice: self tolerance at the level of determinant selection. Eur J Immunol 1992, 22:1819-1825.
15. Myers LK, Seyer JM, Stuart JM, Terato K, David CS, Kang AH: T cell epitopes of type II collagen that regulate murine collagen-induced arthritis. J Immunol 1993, 151:500-505.
16. Basu D, Horvath S, Matsumoto I, Fremont DH, Allen PM: Molecular basis for recognition of an arthritic peptide and a foreign epitope on distinct MHC molecules by a single TCR. J Immunol 2000, 164:5788-5796.
17. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK: Reciprocal development pathways for the generation of pathogenic effector T$_H$17 and regulatory T cells. Nature 2006, 441:235-238.
18. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT: Transforming growth factor-beta induces development of the T (H) 17 lineage. Nature 2006, 441:231-234.
19. He D, Wu L, Kim HK, Li H, Elmets CA, Xu H: CD8+ IL-17 producing T cells are important effector functions for the elicitation of contact hypersensitivities responses. J Immunol 2006, 177:6852-6858.

20. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, Wong CH, Schneider E, Dy M, Leite-de-Moraes MC: Identification of an IL-17-producing NK1.1neg iNKT cell population involved in airway neutrophilia. *J Exp Med* 2007, 204:995-1001.

21. Yoshiga Y, Goto D, Segawa S, Ohnishi Y, Matsumoto I, Ito S, Tsutsumi A, Taniguchi M, Sumida T: Invariant NKT cells produce IL-17 through IL-23-dependent and -independent pathways with potential modulation of Th17 response in collagen-induced arthritis. *Int J Mol Med* 2008, 22:369-374.

22. Lockhart E, Green AM, Flynn JL: IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol* 2006, 177:4662-4669.

23. Matsumoto I, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, Mathis D, Benoist C: How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat Immunol* 2002, 3:360-365.

24. Ranges GE, Sriram S, Cooper SM: Prevention of type II collagen-induced arthritis by in vivo treatment with anti-L3T4. *J Exp Med* 1985, 162:1105-1110.

25. Williams RO, Whyte A: Anti-CD4 monoclonal antibodies suppress murine collagen-induced arthritis only at the time of primary immunization. *Cell Immunol* 1996, 170:291-295.

26. Terato K, Harper DS, Griffiths MM, Hasty DL, Ye XJ, Cremer MA, Seyer JM: Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 1995, 22:137-147.

27. Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K: Extent of T cell receptor ligation can determine the functional differentiation of naïve CD4+ T cells. *J Exp Med* 1995, 182:1591-1596.

28. Leitenberg D, Boutin Y, Constant S, Bottomly K: CD4 regulation of TCR signaling and T cell differentiation following stimulation with peptides of different affinities for the TCR. *J Immunol* 1998, 161:1194-1203.

29. Bockermann R, Schubert D, Kamradt T, Holmdahl R: Induction of a B-cell-dependent chronic arthritis with glucose-6-phosphate isomerase. *Arthritis Res Ther* 2005, 7:R1316-R1324.

30. Critchfield JM, Racke MK, Zúóiga-Pflücker JC, Cannella B, Raine CS, Goverman J, Lenardo MJ: T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science* 1994, 263:1139-1143.

31. Gaur A, Wiers B, Liu A, Rothbard J, Fathman CG: Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science* 1992, 258:1491-1494.

32. Kori Y, Matsumoto I, Zhang H, Yasukochi T, Hayashi T, Iwanami K, Goto D, Ito S, Tsutsumi A, Sumida T: Characterisation of Th1/Th2 type, glucose-6-phosphate isomerase reactive T cells in the generation of rheumatoid arthritis. *Ann Rheum Dis* 2006, 65:968-969.

Research article

Therapeutic effects of antibodies to tumor necrosis factor-α, interleukin-6 and cytotoxic T-lymphocyte antigen 4 immunoglobulin in mice with glucose-6-phosphate isomerase induced arthritis

Isao Matsumoto[1,2], Hua Zhang[1,2], Takanori Yasukochi[1,2], Keiichi Iwanami[1], Yoko Tanaka[1], Asuka Inoue[1], Daisuke Goto[1], Satoshi Ito[1], Akito Tsutsumi[1] and Takayuki Sumida[1]

[1]Division of Clinical Immunology, Major of Advanced Biomedical Applications, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai, Tsukuba 305-8575, Japan
[2]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

Corresponding author: Isao Matsumoto, ismatsu@md.tsukuba.ac.jp

Abstract

Introduction Immunization with glucose-6-phosphate isomerase (GPI) induces severe arthritis in DBA/1 mice. The present study was designed to identify the cytokines and co-stimulatory molecules involved in the development of GPI-induced arthritis.

Methods Arthritis was induced in DBA/1 mice with 300 μg human recombinant GPI. CD4+ T cells and antigen-presenting cells from splenocytes of arthritic mice were cultured in the presence of GPI. Tumor necrosis factor (TNF)-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12 levels were assessed using cytometric bead array. Monoclonal antibodies to TNF-α, IFN-γ, IL-12, CD40L, inducible co-stimulator (ICOS), and cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4Ig) were used to block TNF-α and IFN-γ production, examine clinical index in mice with GPI-induced arthritis, and determine anti-GPI antibody production.

Results Large amounts of TNF-α and IFN-γ and small amounts of IL-2 and IL-6 were produced by splenocytes from mice with GPI-induced arthritis. Anti-TNF-α mAbs and CTLA-4Ig suppressed TNF-α production, whereas anti-IFN-γ mAbs, anti-IL-12 mAbs, and CTLA-4 Ig inhibited IFN-γ production. A single injection of anti-TNF-α and anti-IL-6 mAbs and two injections of CTLA-4Ig reduced the severity of arthritis in mice, whereas injections of anti-IFN-γ and anti-IL-12 mAbs tended to exacerbate arthritis. Therapeutic efficacy tended to correlate with reduction in anti-GPI antibodies.

Conclusion TNF-α and IL-6 play an important role in GPI-induced arthritis, whereas IFN-γ appears to function as a regulator of arthritis. Because the therapeutic effects of the tested molecules used in this study are similar to those in patients with rheumatoid arthritis, GPI-induced arthritis appears to be a suitable tool with which to examine the effect of various therapies on rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder with variable disease outcome, and is characterized by a polyarticular inflammatory process of unknown etiology. The prognosis for RA patients has improved significantly in recent years following the introduction of tumor necrosis factor (TNF)-α antagonists [1]. Despite the increased popularity of this form of therapy, its precise mechanism of action in RA remains unclear.

Collagen-induced arthritis (CIA) is widely used as an experimental model to evaluate the effects of therapeutic agents on human RA. The effects of various anti-cytokine mAbs have been examined in this model, especially after the onset of

AP = alkaline phosphatase; APC = antigen-presenting cell; CBA = cytometric bead array; CIA = collagen-induced arthritis; CTLA-4Ig = cytotoxic T-lymphocyte antigen 4 immunoglobulin; GPI = glucose-6-phosphate isomerase; GST = glutathione S-transferase; hGPI = recombinant GPI-GST fusion; ICOS = inducible co-stimulator; IFN = interferon; IL = interleukin; mAb = monoclonal antibody; PBS = phosphate-buffered saline; RA = rheumatoid arthritis; TNF = tumor necrosis factor.

clinical arthritis. Previous studies reported that anti-IL-1 and anti-IL-12 mAbs significantly suppressed arthritis, whereas anti-TNF-α therapy had little effect in this model [2-5], and blockade of IL-6 had no effect in established CIA [6], indicating different therapeutic mechanisms in RA [7,8].

The ubiquitously expressed self-antigen glucose-6-phosphate isomerase (GPI) was identified as an arthritogenic target in the K/B × N T-cell receptor transgenic mouse model [9,10]. Recently, immunization with human GPI was reported to provoke acute, severe arthritis in DBA/1 mice (GPI-induced arthritis), supporting the notion that T-cell and B-cell responses to GPI play a crucial role in the development of arthritis [11,12]. We recently described the presence of GPI-reactive T cells in HLA-DRB1*0405/*0901-positive patients with RA who harbored anti-GPI antibodies, a finding that emphasizes the pathogenic role of antigen-specific T cells in anti-GPI antibody-positive patients [13].

The aim of the present study was to determine the mechanism of antigen-specific arthritis. For this purpose, we analyzed the role of several cytokines and co-stimulatory molecules in GPI-induced arthritis after clinical onset. The production of TNF-α by cultured splenocytes was increased, and anti-TNF-α mAb and cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4Ig) efficiently suppressed TNF-α production by splenocytes. Furthermore, a single injection of anti-TNF-α mAb and two injections (on days 8 and 12, or days 12 and 16) of CTLA-4Ig markedly reduced the severity of the disease. In contrast, neither anti-IFN-γ nor anti-IL-12 mAb altered the course of the disease. Surprisingly, a single injection of anti-IL-6 mAb resulted in cure of arthritis. Further analyses showed the presence of high serum TNF-α and IL-6 levels, but not IFN-γ and IL-1β, in arthritic mice. Moreover, effective treatment with these agents tended to reduce anti-GPI antibody production. These findings suggest that TNF-α and IL-6 play important roles in acute-onset arthritis in GPI-immunized mice. These results point to the potential roles played by these cytokines in the pathogenicity of human RA, and suggest that therapeutic strategies directed against TNF-α and IL-6 might be fruitful in RA.

Materials and methods
GPI-induced arthritis in DBA/1 mice
Male DBA/1 mice (aged 6 to 8 weeks) were obtained from Charles River (Yokohama, Japan). Recombinant human GPI was prepared as described previously [14]. Mice were immunized by intradermal injection of 300 µg recombinant human GPI-glutathione S-transferase (GST) fusion protein (hGPI) in emulsified complete Freund's adjuvant (Difco, Detroit, MI, USA). Control mice were immunized with 300 µg GST in complete Freund's adjuvant. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Tsukuba University School of Medicine. Arthritic animals were clinically assessed and ankle thickness recorded. We used the following arthritis scoring system to

evaluate the disease state (clinical index): 0 = no evidence of inflammation, 1 = subtle inflammation or localized edema, 2 = easily identified swelling but localized to either dorsal or ventral surface of paws, and score 3 = swelling on all aspects of paws. All four limbs were evaluated using a constant tension caliper and graded, yielding a maximum possible score of 12 per mouse.

Histological assessment of arthritis
At the indicated time points, the ankles of the mice were removed, fixed, decalcified and paraffin-embedded. Sections (5 µm) were stained with hematoxylin and eosin, and evaluated for histologic changes indicating inflammation, pannus formation, and cartilage and bone damage.

Preparation of splenocytes and cytometric bead array
Spleens were dissected from immunized DBA/1 or B6 mice (on day 8 after immunization) and immediately immersed in phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA). Single-cell suspensions were prepared. Red blood cells were lysed by incubation of the suspension in NH$_4$Cl (0.83% in 0.01 mol/l Tris-HCl [pH 7.2]). The number of splenocytes was then counted, centrifuged again, and resuspended in RPMI (Gibco, Grand Island, NY, USA). For culture, we used RPMI supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 10% fetal bovine serum, and 50 µM 2-mercaptoethanol. After counting the cells, the medium was added to make the final concentration 2.5 × 10^6/ml. Next, CD4+ T cells were isolated by positive selection with anti-mouse CD4+ antibody (T cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). The labeled cells were then passed through separation columns (MiniMACS columns; Miltenyi Biotec). The cells contained more than 97% CD4+ T cells. T-depleted spleen cells were treated with 50 µg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) for 30 minutes at 37°C and were used as antigen-presenting cells (APCs).

CD4+ T cells (1 × 10^6 cells/ml) were stimulated with 5 µg/ml GPI (or GST) and APCs (2 × 10^5 cells/ml) in 1 ml volume in 48-well culture plates (Nunc) for 12 hours. The culture supernatants were collected and cell-free samples were stored at -30°C until the cytokine assay. The concentrations of TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were measured using cytometric bead array (CBA) with a series of anti-cytokine mAb-coated beads and PE-conjugated anti-cytokine mAbs, followed by Epics XL flow cytometric analysis (Beckman-Coulter Electronics, Fullerton, CA, USA), using the CBA kit (BD Bioscience, San Jose, CA, USA) and software (BD).

Antibodies used for in vitro and in vivo studies
We used commercially available anti-TNF-α mAb (eBioscience, San Diego, CA, USA; 10 µg/ml), anti-IFN-γ mAb (BD Biosciences; 1 µg/ml), and anti-IL-12 mAb (BD; 0.3 µg/ml) to neutralize the respective cytokines. These concentrations were selected based on more than 80% blockade of the

respective cytokine. CTLA-4Ig (BD; 1 μg/ml), anti-inducible co-stimulator (ICOS) mAb (BD; 0.5 μg/ml), and anti-CD40L mAb (BD; 1 μg/ml) were used to block co-stimulatory pathways. As a control antibody, we used the same amount of rat IgG₁ isotype control (R&D Systems, Minneapolis, MN, USA). Inhibition study was conducted by adding the above concentration at the start of culture. Three independent experiments were performed.

On day 8 after the onset of arthritis, each mouse received a single injection of 100 μg of anti-TNF-α mAb, anti-IL-12 mAb, anti-IFN-γ mAb or anti-IL-6 mAb. A single injection of anti-IL-6 mAb on day 14 was also administered. On the other hand, two injections of 100 μg CTLA-4Ig were administered on days 8 and 12, or on days 12 and 16 after the onset of arthritis.

Measurement of serum levels of cytokines and anti-GPI antibodies

Serum samples were collected at the indicated time points. The serum levels of TNF-α, IL-6, IL-1β and IFN-γ were determined with the respective enzyme-linked immunosorbent assay kits (BD). To detect the levels of anti-GPI antibodies, we used hGPI and GST at 5 μg/ml (diluted in PBS) to coat microtiter plates (12 hours, 4°C). After washing twice with washing buffer (0.05% Tween 20 in PBS), Block Ace (diluted 1/4 in 1 × PBS; Dainippon Pharmaceuticals, Osaka, Japan) was used for saturation (2 hours at room temperature). After two washes, sera (diluted 1/500) were added and the plates incubated for 2 hours at room temperature. After washing, alkaline phosphatase (AP)-conjugated anti-mouse IgG (Fc-fragment specific; Jackson Immunoresearch Laboratories, West Grove, PA, USA) was added to the plate (dilution 1/5,000, 1 hour, room temperature). After three washes, color was developed with AP reaction solution (containing 9.6% diethanolamine and 0.25 mmol/l MgCl₂ [pH 9.8]) with AP substrate tablets (Sigma Chemical Co., St. Louis, MO, USA; one AP tablet per 5 ml AP reaction solution). Plates were incubated for 30 minutes at room temperature and the optical density was measured by plate spectrophotometry at 405 nm. Determinations were conducted in triplicate, and standardized between experiments by reference to a highly positive mouse anti-GPI serum. The primary reading was processed by subtracting optical density readings of control wells (coated with GST for hGPI).

Statistical analysis

All data were expressed as mean ± standard error of the mean. Differences between groups were examined for statistical significance by using Mann-Whitney's U test. P < 0.05 denoted the presence of a statistically significant difference.

Results

Induction of arthritis in mice immunized by recombinant human GPI

To investigate whether our own GPI immunization procedure can induce arthritis, we immunized DBA/1 mice using human
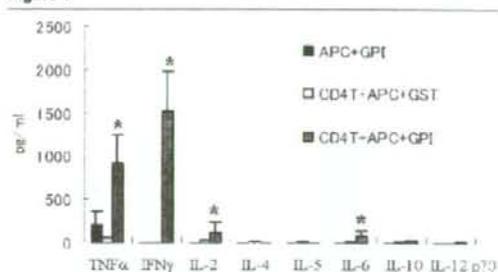
GPI-induced TNF-α and IFN-γ production from arthritic splenocytes *in vitro*. Spleens were removed from glucose-6-phosphate isomerase (GPI)-immunized DBA/1 mice (on day 8 after immunization), and then single-cell suspensions were prepared. MACS separated CD4⁺ T cells (1 × 10⁶ cells/ml) were stimulated with 5 μg/ml GPI (or glutathione S-transferase [GST]) and antigen-presenting cells (APCs; 2 × 10⁵ cells/ml, mitomycin treated) for 12 hours. The culture supernatants were collected and concentrations of tumor necrosis factor (TNF)-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were measured by cytometric bead array. Data were averages of three independent experiments. Error bars represent ± standard error. *P < 0.05, by Mann-Whitney U-test.

recombinant GPI prepared in our laboratories. As reported previously [9,10,15], all mice developed arthritis after immunization with 300 μg recombinant GPI. Arthritis appeared at day 8, and severe arthritis was noted at day 14, with maximum ankle swelling on day 14 (data not shown). GST immunization did not induce apparent arthritis (data not shown).

GPI induces production of TNF-α and IFN-γ by spleen cells at onset of arthritis

To identify the dominant cytokines at the onset of antigen-induced arthritis (day 8), we established the CBA array system using spleen CD4⁺ T cells plus mitomycin-treated APCs cultured in GPI. In this system, treatment of APCs with mitomycin is designed to kill autoreactive APCs. The results demonstrated the production of large amounts of TNF-α and IFN-γ by the spleen of arthritic mice (Figure 1). In contrast, cells cultured with control antigen (GST) instead of GPI did not produce these cytokines (Figure 1). APC plus antigen alone produced such amounts of cytokines. Very low but detectable levels of IL-2 and IL-6 were produced, but almost no T-helper-2 type cytokines (such as IL-4, IL-5, and IL-10) were detected (Figure 1). These results indicate that exposure to the GPI antigen results in induction of TNF-α and IFN-γ by immunocytes, and suggest that these cytokines could play a crucial role in the induction of arthritis in GPI-induced mice.

Anti-cytokine mAbs and co-stimulator blockade inhibit *in vitro* cytokine production

To delineate the separate contributions of TNF-α and IFN-γ, we performed blocking experiments using neutralizing mAbs for anti-TNF-α, IFN-γ, and IL-12 using the CBA array system. TNF-α production was inhibited by anti-TNF mAb (64.7 ±

Figure 2
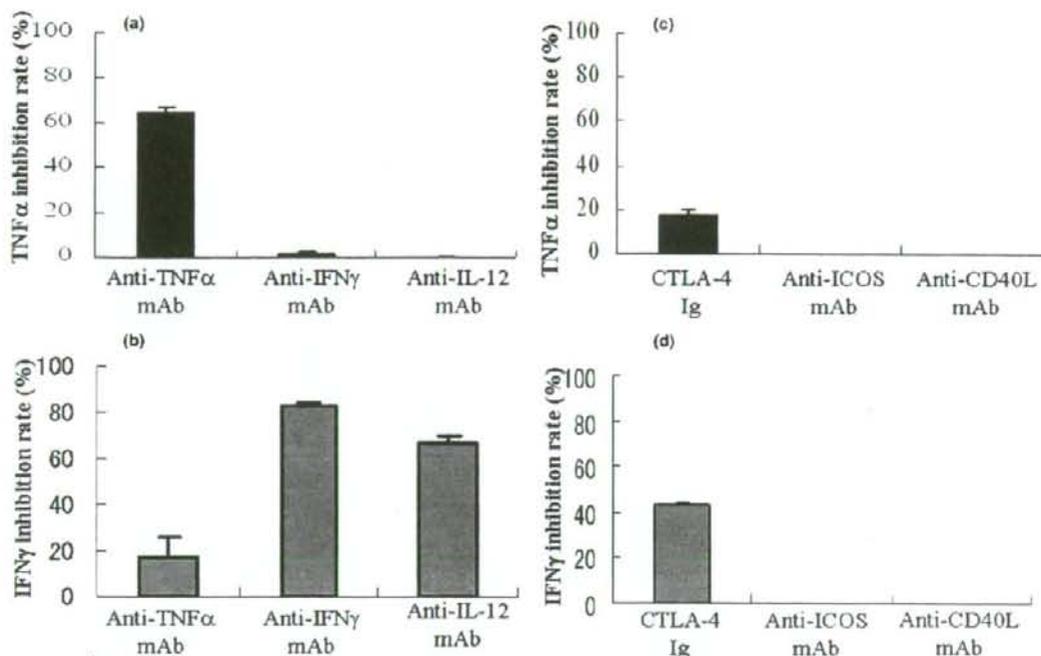
In vitro inhibition assay of GPI-induced TNF-α and IFN-γ production using anti-cytokine mAbs or anti-co-stimulators. High amounts of tumor necrosis factor (TNF)-α and IFN-γ were produced by splenocytes cultured with glucose-6-phosphate isomerase (GPI). Thus, we used anti-TNF-α mAb (10 μg/ml), anti-IFN-γ mAb (1 μg/ml), and anti-IL-12 mAb (0.3 μg/ml) to neutralize these cytokines in the *in vitro* cytometric bead array system. Inhibition study was conducted by adding the above concentrations at commencement of culture. These concentrations were calculated to produce more than 80% blockade of these cytokines. The percentage inhibition rate is calculated by cytokine production with this system: 100 − ([cytokine mAb − control antibody]/control antibody). The inhibition rate of (a) TNFα and (b) IFN-γ are shown. Cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4Ig; 1 μg/ml), anti-inducible co-stimulator (ICOS) mAb (0.5 μg/ml), and anti-CD40L mAb (1 μg/ml) were also used to block co-stimulatory pathways, and the inhibition rate of (c) TNF-α and (d) IFN-γ are shown. Three independent experiments were performed. Data are expressed as mean ± standard error of the mean.

2.7%; Figure 2a), but not by anti-IL-12 mAb (0%; Figure 2a). On the other hand, IFN-γ production was inhibited by anti-IFN-γ mAb (82.5 ± 1.2%; Figure 2b) as well as by anti IL-12 mAb (67.5 ± 2.5%; Figure 2b), and weakly by anti-TNF-α mAb (17.2 ± 9.2%; Figure 2b). These results suggest that TNF-α production is not regulated by IFN-γ, although IFN-γ is partially regulated by TNF-α.

To determine the effect of co-stimulatory molecules in established arthritis, we conducted the same *in vitro* experiments by using CTLA-4Ig, anti-ICOS, and anti-CD40L mAbs. CTLA-4Ig suppressed TNF-α (18 ± 2.1%; Figure 2c), and IFN-γ (42.9 ± 2.1%; Figure 2d) production, but not anti-ICOS or anti-CD40L mAb. These findings suggest that the antigen-induced cytokines are mainly driven by CD28/B7-1,2 co-stimulator.

Treatment of GPI-induced arthritis with anti-TNF-α mAb

To identify the pathogenic cytokine that can provoke the onset of arthritis, we conducted *in vivo* experiments using neutralizing mAbs. A single injection of 100 μg of anti-TNF-α mAb at day 8 ameliorated the disease (Figure 3a). In contrast, injection of the same dose of anti-IFN-γ or anti-IL-12 mAb had no such effect on the course of the disease, but rather tended to exacerbate the arthritis (Figure 3b). Histopathological examination of the joints of treated mice showed a clear therapeutic effect for anti-TNF-α mAb (Figure 3f, on day 21) as compared with that of control antibody (Figure 3g, on day 21). These results suggest that TNF-α blockade has clear therapeutic effect in GPI-induced model, irrespective of the minor role of 'conventional' T-helper-1 autoimmunity.

Figure 3
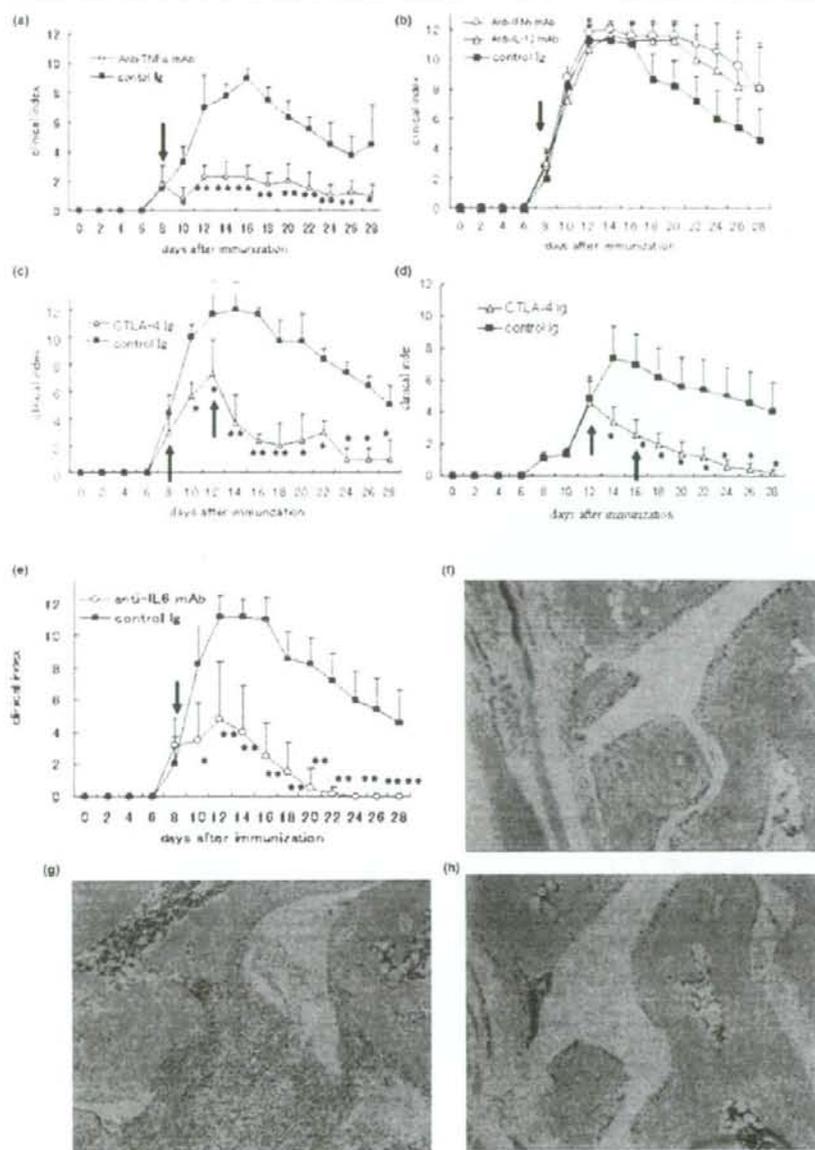
Therapeutic effect of anti-TNF mAb, CTLA-4Ig, and anti-IL-6 mAb in GPI-induced arthritis. Glucose-6-phosphate isomerase (GPI)-immunized mice were treated with (a) anti-tumor necrosis factor (TNF)-α mAb; (b) anti-IFN-γ mAb or anti-IL-12 mAb; (c) cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4Ig; on days 8 and 12); (d) CTLA-4Ig (on days 12 and 16); and (e) or anti-IL-6 mAb just after the onset of arthritis (on day 8, on days 8 and 12, or days 12 and 16; arrow). The mean clinical index (± standard error) was examined throughout the study. *P < 0.05, **P < 0.01, by Mann-Whitney's U test. n = 6 mice in each group. Hematoxylin and eosin staining at day 21 (×40) is also shown: (f) anti-TNF-α mAb, (g) control antibody, and (h) CTLA-4Ig (on days 8 and 12).

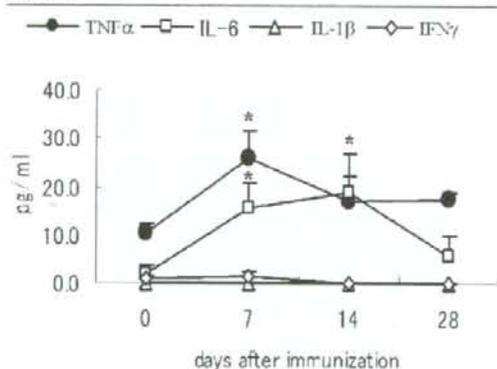### Treatment of GPI-induced arthritis with CTLA-4Ig and anti-IL-6 mAb

To investigate the effect of CTLA-4Ig *in vivo*, we treated arthritic mice with CTLA-4Ig on days 8 and 12, or on days 12 and 14. A marked improvement was seen after the second treatment (on days 8 and 12), probably because of a reduction in effector T cells at that stage (Figure 4c, and hematoxylin and eosin staining on day 21 in Figure 4h). Moreover, if we admininstered treatment on days 12 and 16, clear therapeutic efficacy was observed after the first treatment. This finding suggests that CTLA-4Ig is also therapeutically potent, especially on day 12, in mice with GPI-induced arthritis.

IL-6 is also an important cytokine in arthritis, and it is considered a promising target for the treatment of RA [7,8]. Serum IL-6 concentrations were elevated in arthritic mice, especially during the disease effector phase (Figure 4). In the next step, we assessed the effect of IL-6 blockade in mice with GPI-induced arthritis. Surprisingly, anti-IL-6 treatment on day 8 resulted in improvement in the clinical index (Figure 3e), although treatment on day 14 had no effect on the course of the disease (data not shown), suggesting that IL-6 is also pathologically crucial in the early effector phase in arthritis.

Role of various inflammatory cytokines in GPI-induced arthritis

To determine the effects of inflammatory cytokines during the effector phase of arthritis, we measured the serum concentrations of TNF-α, IL-6, IL-1β, and IFN-γ at days 0, 7, 14, and 28 in DBA/1 mice after GPI immunization. Serum TNF-α concentration was upregulated at disease onset (day 7), but gradually decreased to the basal level by day 28 (Figure 4). On the other hand, serum IL-6 concentration was upregulated gradually, especially during the disease effector phase (days 7 and 14; Figure 4). In contrast, serum IL-1β and IFN-γ concentrations were persistently low and below the detection limit (4 pg/ml) in GPI-induced mice throughout the study (Figure 4). These findings suggest a systemic TNF-α/IL-6 imbalance in arthritic mice.

Effective treatments tend to alter anti-GPI antibody production

Anti-GPI antibodies have potent arthritogenic capacity in K/B × N mice. However, anti-GPI antibodies from mice with GPI-induced arthritis do not solely cause arthritis (Schubert and coworkers [11] and our preliminary observations). In GPI-induced arthritis, IgG and C3 are co-localized on the articular surface of arthritic joints (Tanaka and coworkers, unpublished data). Accordingly, we compared the effects of anti-cytokine mAbs, immunomodulatory molecule CTLA-4Ig, and control immunoglobulin on the production of anti-GPI antibodies in mice with GPI-induced arthritis. The antigen was injected on day 8, and then sera were collected on day 14. As shown in Figure 5, anti-TNF-α, anti-IL-6, and CTLA-4Ig tended to suppress the production of anti-GPI antibodies, whereas IL-12 mAb slightly enhanced the production of the antibodies. These findings suggest that effective treatments might also alter autoantibody production during this phase of GPI-induced arthritis.
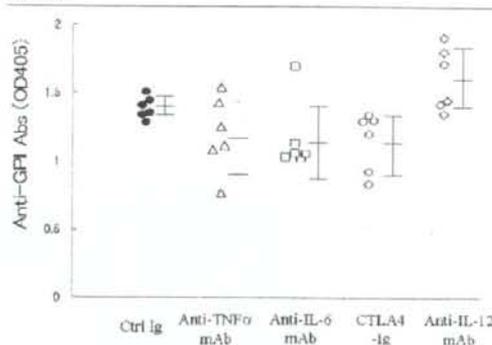
Figure 4

Concentration of inflammatory cytokines in serum of mice with GPI-induced arthritis. After immunization with glucose-6-phosphate isomerase (GPI), serum samples were collected from GPI-induced DBA/1 mice at the indicated time points (days 0, 7, 14, and 28). Serum concentrations of tumor necrosis factor (TNF)-α (solid circle), IL-6 (open square), IL-1β (open triangle), or IFN-γ (open diamond) were determined by enzyme-linked immunosorbent assay. Data are expressed as mean ± standard error. n = 3 mice in each group. *P < 0.05, by Mann-Whitney's U-test.

Figure 5

Effective treatments tend to alter anti-GPI antibody production. Glucose-6-phosphate isomerase (GPI)-induced arthritic mice were treated with 100 µg anti-tumor necrosis factor (TNF)-α mAb, anti-IL-6 mAb, cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4Ig), and anti-IL-12 mAb on day 8, and CTLA-4 Ig on day 12. Serum samples were collected on day 14. The titers of anti-GPI antibodies were analyzed by enzyme-linked immunosorbent assay. Each symbol represents a single animal. Data are expressed as mean ± standard deviation of optical density.

Discussion

GPI, a ubiquitous glycolytic enzyme, is a new candidate autoantigen in inflammatory arthritis, initially identified in K/B × N mice [10]. In K/B × N mice, anti-GPI antibodies solely induce arthritis through activation of complements and Fcγ receptors [16]. With regard to cytokine dependency, anti-TNF-α mAb does not prevent the development of arthritis in K/B × N mice, and IL-6 deficiency has no influence on the development of arthritis by K/B × N serum transfer [17]. Based on this cytokine dependency, K/B × N mice differ from patients with RA.

Although the therapeutic effect of TNF antagonists has been established in RA, there are few animal models of arthritis in which TNF antagonists are confirmed as being therapeutically beneficial. For example, in the most conventional RA models, such as CIA, treatment with IL-1 antagonists significantly suppressed arthritis, whereas TNF antagonists had minor effect [2-4]. On the other hand, a clear therapeutic effect of anti-TNF-α mAb was reported recently in DNaseII-type I IFN double knockout mice [18], although this was not a genetically unaltered mouse. Schubert and coworkers [11] reported that continuous injections of human TNF receptor p75-IgG-Fc fusion protein (etanercept) from days 0 to 9 completely protected against the development of arthritis in GPI-induced arthritis. In this regard, we demonstrated a clear therapeutic effect for TNF antagonist in mice with GPI-induced arthritis, and the therapeutic response correlated with the in vitro regulation of TNF production. For example, we detected specific TNF-α-induced molecules in spleen and joints of mice with GPI-induced arthritis by Genechip analysis (Matsumoto and Inoue, unpublished data). These results also indicate that the GPI-induced arthritis model is suitable tool for studying the mechanisms of action of TNF-α antagonists in RA patients.

CTLA-4Ig can selectively modulate the CD80 or CD86-CD28 co-stimulatory signal required for full T-cell activation [19], and is a promising new molecule for treatment of RA [19-21]. Although administration of CTLA-4Ig at the time of immunization prevented the development of CIA, the therapeutic efficacy has not been clearly confirmed in this model [22]. In the present study, we demonstrated that only two injections of CTLA-4Ig (both on days 8 and 12 or on days 12 and 16) markedly prevented the development of arthritis in mice with GPI-induced arthritis. What is the mechanism of action of CTLA-4Ig in GPI-induced arthritis? We recently reported that anti-IL-17 mAb is also therapeutically promising in this model [15], and thus effector T-helper-17 dependency is much stronger than in the CIA model. The present study showed that treatment with CTLA-4Ig resulted in suppression of anti-GPI antibody production. Therefore, blockade of persistent T-cell activation during the early effector phase appears therapeutically useful in GPI-induced arthritis, through inhibition of both effector T-helper-17 cells and autoantibody production.

Like TNF-α and IL-1, IL-6 is a pleiotropic cytokine that is known to play a role in RA, and a humanized anti-IL-6 receptor antibody (tocilizumab) was recently reported to be beneficial therapeutically [7,8]. However, administration of IL-6 antagonist did not produce any remedial effects when administered after the onset of arthritis in CIA animals [6]. In the present study we demonstrated that treatment with anti-IL-6 mAb inhibited the development of arthritis and even after the onset of arthritis in mice with GPI-induced arthritis. However, anti-IL-6 mAb had no effect on day 14, even if we used 4 mg anti-IL-6 receptor mAb [15]. Our results with the in vitro CBA assay showed that IL-6 was not the main cytokine produced by antigen cultures. Cultures of the same numbers of mitomycin-untreated and -treated splenocytes with GPI showed that IL-6 was predominantly produced by whole spleen cells, indicating that mitomycin-sensitive APCs, including B cells, were the major source of IL-6 (data not shown). Another study showed that IL-6 antagonism on day 8 suppressed the proliferation of antigen-specific T cells and partially the development of T-helper-17 cells, with reduced production of anti-GPI antibody [15]. Therefore, the effectiveness by IL-6 antagonist on day 8 in GPI-induced arthritis appears to be mediated through orchestration of these mechanisms.

In the GPI-induced arthritis model, anti-GPI antibodies could not induce arthritis on their own. Neither Fcγ receptor deficient nor B-cell-deficient mice had overt arthritis [11,12], suggesting that anti-GPI antibodies play an indispensable role in this model. Recent studies identified co-localization of IgG and C3 on the articular surface of joints in GPI-induced arthritis on day 14, and production of anti-GPI antibodies was most vigorous on day 8 (Tanaka and coworkers, unpublished data). These results mimic those of arthritis mediated by K/B × N serum transfer [23]. Thus, we investigated whether immunomodulatory molecules could alter this vigorous antigen-specific antibody production on day 8. Treatment of mice with CTLA-4Ig resulted in downregulation of anti-GPI antibody production, whereas anti-TNF-α and anti-IL-6 mAb therapy tended to reduce these antibodies. In contrast, anti-IL-12 mAb rather upregulated the production of anti-GPI antibodies, leading to persistent arthritis. These findings suggest that production of anti-GPI antibodies in the early effector phase may correlate with the severity of arthritis in this model.

Does this model mimic human RA, especially in anti-GPI antibody-positive individuals? Severe forms of RA have been described in patients with high titers of anti-GPI antibodies, although these antibodies were also identified in a few control individuals [14,24]. In anti-GPI antibody-positive individuals, GPI-reactive CD4+ T cells, especially T-helper-1 type cells, were detected among peripheral blood mononuclear cells of RA patients with either HLA-DR 0405 or 0901 haplotype [13]. What about GPI-induced arthritis? High titers of anti-GPI antibodies are present in arthritis-resistant C57BL/6 mice (H-2b) [11,12], although the T cells of these animals exhibited weak

GPI responses compared with arthritis-susceptible DBA/1 mice (H-2^q). These results indicate that anti-GPI antibodies cannot themselves induce arthritis; it is likely that a unique H-2 haplotype and activation of antigen-specific T cells are necessary for the development of arthritis in this model. Moreover, the effectiveness of CTLA-4Ig was clearly similar to that in human RA. Considered together, GPI-induced arthritis seems to be akin to human RA.

Conclusion
Because the therapeutic effects of the tested biologics used in this study are similar to those in patients with RA, GPI-induced arthritis is a suitable model for examining the pathogenic mechanisms of RA and the effect of various treatments.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
IM wrote the manuscript and conceived of the study. HZ, TY, KI, YT, and AI performed all experiments and coordinated the statistical study. TH participated in clinical assessment. TS participated in its full design and coordination, and DG, SI and AT participated in discussions.

Acknowledgements

References
1. Feldmann M, Brennan F, Foxwell BM, Taylor PC, Williams RO, Maini RN: Anti-TNF therapy: where have we got to in 2005? *J Autoimmun* 2005, 25:26-28.
2. Joosten LA, Helsen MM, Saxne T, Loo FA van De, Heinegard D, Berg WB van Den: IL-1 α β blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-α blockade only ameliorates joint inflammation. *J Immunol* 1999, 163:5049-5055.
3. Joosten LA, Helsen MM, Loo FA van de, Berg WB van den: Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF α, anti-IL-1 α/β, and IL-1Ra. *Arthritis Rheum* 1996, 39:797-809.
4. Williams RO, Marinova-Mutafchieva L, Feldmann M, Maini RN: Evaluation of TNF-α and IL-1 blockade in collagen-induced arthritis and comparison with combined anti-TNF-α/anti-CD4 therapy. *J Immunol* 2000, 165:7240-7245.
5. Butler DM, Malfait AM, Maini RN, Brennan FM, Feldmann M: Anti-IL-12 and anti-TNF antibodies synergistically suppress the progression of murine collagen-induced arthritis. *Eur J Immunol* 1999, 29:2205-2212.
6. Takagi N, Mihara M, Moriya Y, Nishimoto N, Yoshizaki K, Kishimoto T, Takeda Y, Ohsugi Y: Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. *Arthritis Rheum* 1998, 41:2117-2121.
7. Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J, Kishimoto T: Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum* 2004, 50:1761-1769.
8. Smolen JS, Maini RN: Interleukin-6: a new therapeutic target. *Arthritis Res Ther* 2006, 8(suppl 2):S5.
9. Kouskoff V, Korganow A-S, Duchatelle V, Degott C, Benoist C, Mathis D: Organ-specific disease provoked by systemic autoreactivity. *Cell* 1996, 87:811-822.
10. Matsumoto I, Staub A, Benoist C, Mathis D: Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science* 1999, 286:1732-1735.
11. Schubert D, Maier B, Morawietz L, Krenn V, Kamradt T: Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in genetically unaltered mice. *J Immunol* 2004, 172:4503-4509.
12. Bockermann R, Schubert D, Kamradt T, Holmdahl R: Induction of a B-cell-dependent chronic arthritis with glucose-6-phosphate isomerase. *Arthritis Res Ther* 2005, 7:1316-1324.
13. Kori Y, Matsumoto I, Zhang H, Yasukochi T, Hayashi T, Iwanami K, Goto D, Ito S, Tsutsumi A, Sumida T: Characterisation of Th1/Th2 type, glucose-6-phosphate isomerase reactive T cells in the generation of rheumatoid arthritis. *Ann Rheum Dis* 2006, 65:968-969.
14. Matsumoto I, Lee DM, Goldbach-Mansky R, Sumida T, Hitchon CA, Schur PH, Anderson RJ, Coblyn JS, Weinblatt ME, Brenner M, Duclos B, Pasquali JL, El-Gabalawy H, Mathis D, Benoist C: Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and spectrum of other chronic autoimmune disorders. *Arthritis Rheum* 2003, 48:944-954.
15. Iwanami K, Matsumoto I, Tanaka-Watanabe Y, Inoue A, Mihara M, Ohsugi Y, Mamura M, Goto D, Ito S, Tsutsumi A, Kishimoto T, Sumida T: Crucial role of IL-6/IL-17 cytokine axis in the induction of arthritis by glucose-6-phosphate isomerase. *Arthritis Rheum* 2008, 58:754-763.
16. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, Takahashi K, Holers VM, Walport M, Gerard C, Ezekowitz A, Carroll MC, Brenner M, Weissleder R, Verbeek JS, Duchatelle V, Degott C, Benoist C, Mathis D: Arthritis critically dependent on innate immune system players. *Immunity* 2002, 16:157-168.
17. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, Gravallese E, Mathis D, Benoist C: Critical roles for interleukin 1 and tumor necrosis factor α in antibody-induced arthritis. *J Exp Med* 2002, 196:77-85.
18. Kawane K, Ohtani M, Miwa K, Kizawa T, Kanbara Y, Yoshioka Y, Yoshikawa H, Nagata S: Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* 2006, 443:998-1002.
19. Ruderman EM, Pope RM: Drug Insight: abatacept for the treatment of rheumatoid arthritis. *Nat Clin Pract Rheumatol* 2006, 2:654-660.
20. Kremer JM, Westhovens R, Leon M, Di Giorgio E, Alten R, Steinfeld S, Russell A, Dougados M, Emery P, Nuamah IF, Williams GR, Becker JC, Hagerty DT, Moreland LW: Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med* 2003, 349:1907-1915.
21. Genovese MC, Becker JC, Schiff M, Luggen M, Sherrer Y, Kremer J, Birbara C, Box J, Natarajan K, Nuamah I, Li T, Aranda R, Hagerty DT, Dougados M: Abatacept for rheumatoid arthritis refractory to tumor necrosis factor α inhibition. *N Engl J Med* 2005, 353:1114-1123.
22. Webb LM, Walmsley MJ, Feldmann M: Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 costimulatory pathway: requirement for both B7-1 and B7-2. *Eur J Immunol* 1996, 26:2320-2328.
23. Matsumoto I, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, Mathis D, Benoist C: How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat Immunol* 2002, 3:360-365.
24. van Gaalen FA, Toes RE, Ditzel HJ, Schaller M, Breedveld FC, Verweij CL, Huizinga TW: Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis. *Arthritis Rheum* 2004, 50:395-399.

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

Keiichi Iwanami,[1] Isao Matsumoto,[2] Yoko Tanaka-Watanabe,[1] Asuka Inoue,[1]
Masahiko Mihara,[3] Yoshiyuki Ohsugi,[4] Mizuko Mamura,[1] Daisuke Goto,[1] Satoshi Ito,[1]
Akito Tsutsumi,[1] Tadamitsu Kishimoto,[5] and Takayuki Sumida[1]

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 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 found to be 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. Administration of MR16-1 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 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 after arthritis onset rapidly ameliorates the arthritis, 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 overnight at 37°C before the addition of 0.1 mM 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
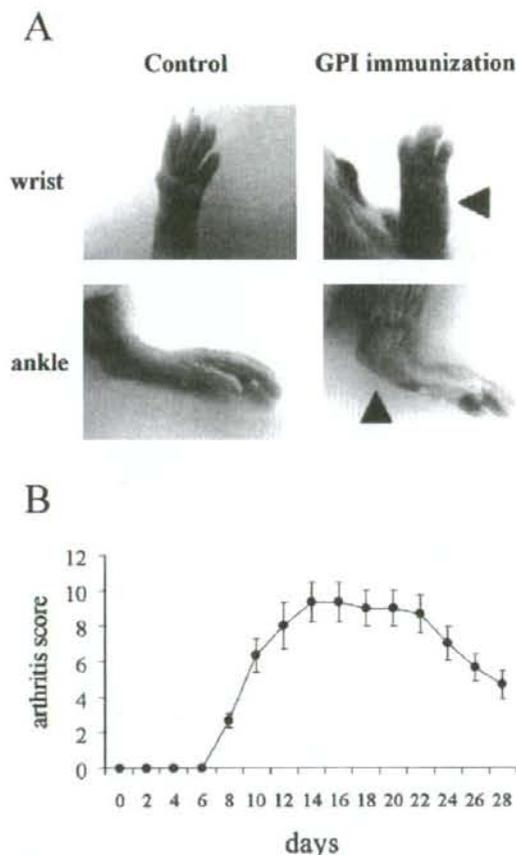

Figure 1. Induction of severe polyarthritis by immunization with recombinant human glucose-6-phosphate isomerase (GPI). DBA/1 mice were immunized with 300 μg of recombinant human GPI, and the development of arthritis was monitored visually and scored on a scale of 0–3 (see Materials and Methods for details). 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.

that was localized to either the dorsal or ventral surface of the paw, and 3 = swelling of all aspects of the paw.

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$_4$Cl, 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 μM 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 $\mu g/ml$ of mitomycin C were used as antigen-presenting cells (APCs). Purified CD4+ T cells and APCs were cocultured with 5 $\mu g/ml$ 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 $\mu g/ml$ 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 mM $MgCl_2$, 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 × 10⁶/ml) were stimulated with 100 $\mu g/ml$ of recombinant human GPI in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) for 24 hours. GolgiStop (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 Treg cell staining kit with forkhead box P3 (FoxP3) (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).

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 × 10⁷/ml) in PBS were stained with 1.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Molecular Probes, Eugene, OR) for 8 minutes. Stained cells were cultured with 25 $\mu g/ml$ of recombinant human GPI at 1 × 10⁶/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
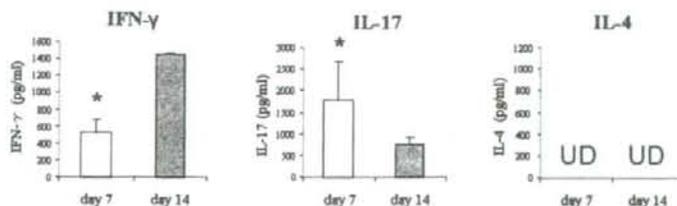
Figure 2. Differentiation of glucose-6-phosphate isomerase (GPI)–specific CD4+ T cells into Th1 and Th17 cells. CD4+ T cells and mitomycin C–treated antigen-presenting cells were stimulated for 24 hours with GPI on either day 7 (induction phase) or day 14 (effector phase) and then assessed for the production 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).
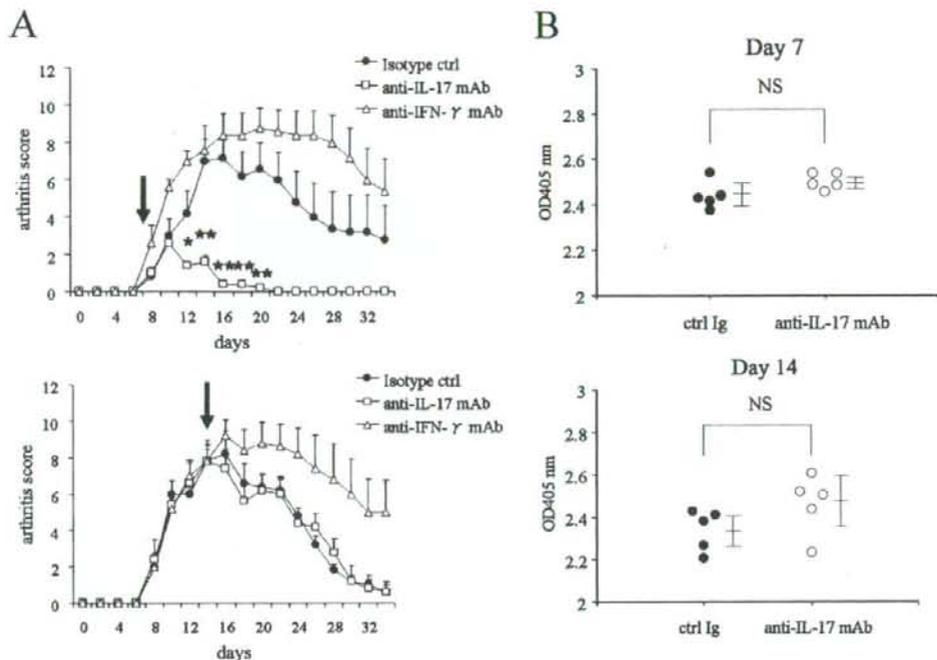
Figure 3. Suppression of the development of glucose-6-phosphate isomerase (GPI)–induced arthritis by treatment with anti–interleukin-17 (anti–IL-17) monoclonal antibody (mAb). **A,** Arthritis scores following intraperitoneal injection of 100 μg of anti–IL-17 mAb or anti–interferon-γ (anti-IFNγ) mAb 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).

days 7–8, showed peak severity on day 14, then gradually subsided (Figure 1B).

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 with recombinant 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 ($P < 0.01$), 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).
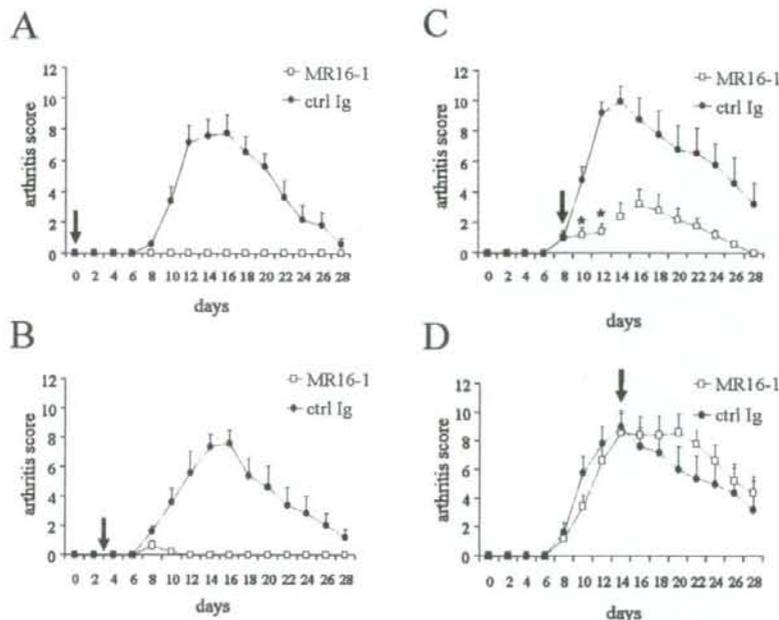
Figure 4. Inhibition of the development of arthritis by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 or control Ig on day 0 (**A**), 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 versus controls, 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 in 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 (data not shown).
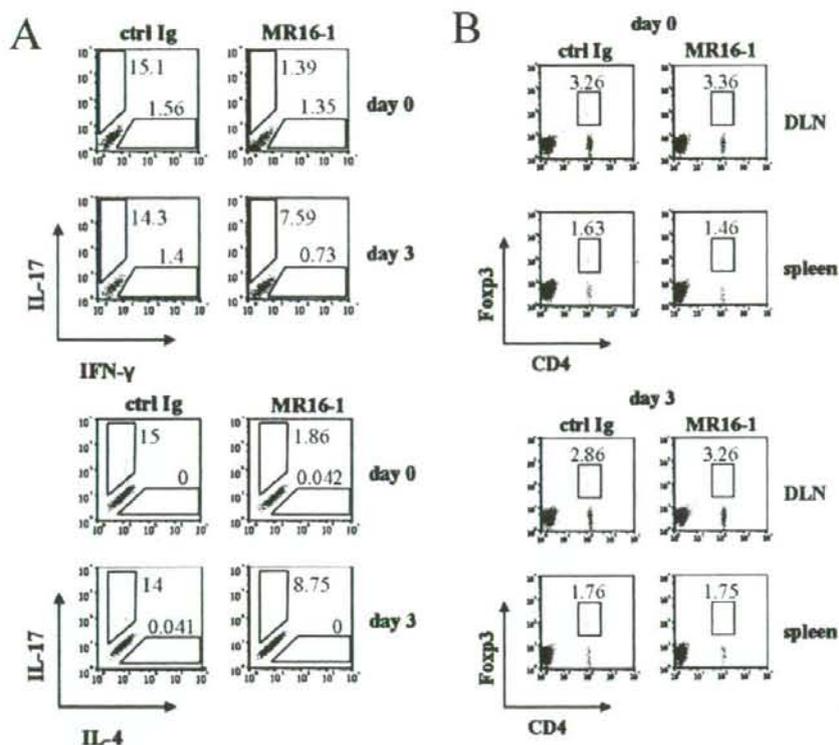
Figure 5. Inhibition of the differentiation of draining lymph node cells into Th17 cells by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 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. GolgiStop 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 dot plots are the percentages of positive cells in the compartment.

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).

We also used intracellular staining methods to examine 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
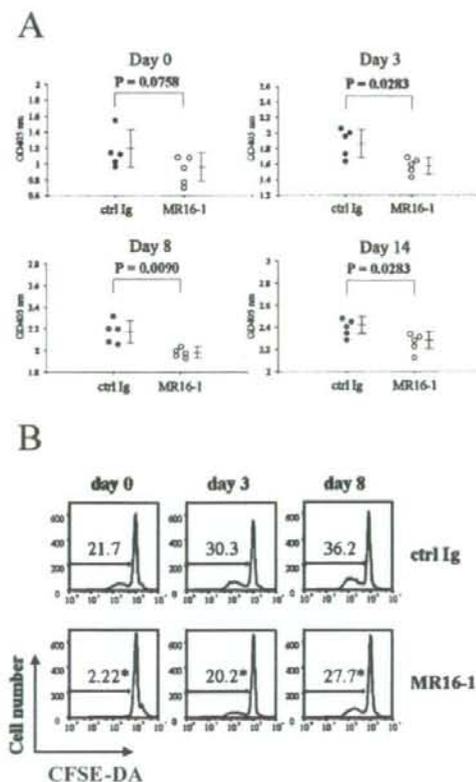
A

B

CFSE-DA

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 the anti–IL-6R 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.

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.

DISCUSSION

GPI, a ubiquitous glycolytic enzyme, is a new candidate autoantigen in the initiation of autoimmune arthritis (2). 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 (11). 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 a T cell–dependent manner. 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 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 ana-

lysis 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

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.

REFERENCES

1. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoreactivity. Cell 1996;87:811–22.

2. Matsumoto I, Staub A, Benoist C, Mathis D. Arthritis provoked by linked T and B recognition of a glycolytic enzyme. Science 1999;286:1732–5.

3. Schubert D, Maier B, Morawietz L, Krenn V, Kamradt T. Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in generally unaltered mice. J Immunol 2004;172:4503–9.

4. Ranges GE, Sriram S, Cooper SM. Prevention of type II collagen-induced arthritis by in vivo treatment with anti-L3T4. J Exp Med 1985;162:1105–10.

5. Williams RO, Whyte A. Anti-CD4 monoclonal antibodies suppress murine collagen-induced arthritis only at the time of primary immunization. Cell Immunol 1996;170:291-5.

6. Tada Y, Ho A, Koh DR, Mak TW. Collagen-induced arthritis in CD4- or CD8-deficient mice: CD8 T cells play a role in initiation and regulate recovery phase of collagen-induced arthritis. J Immunol 1996;156:4520–6.

7. Matsumoto I, Lee DM, Goldbach-Mansky R, Sumida T, Hitchon CA, Schur PH, et al. Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and a spectrum of other chronic autoimmune disorders. Arthritis Rheum 2003;48:944–54.

8. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal development pathways for the generation of pathogenic effector T_H17 and regulatory T cells. Nature 2006;441:235–8.

9. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-β induces development of the T_H17 lineage. Nature 2006;441:231–4.

10. Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. Stat 3 activation is responsible for IL-6 dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. J Immunol 1998;161:4652–60.

11. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 1999;10:451–61.

12. Kyburz D, Carson DA, Corr M. The role of CD40 ligand and tumor necrosis factor α signaling in the transgenic K/BxN mouse model of rheumatoid arthritis. Arthritis Rheum 2000;43:2571–7.

13. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, et al. Critical roles for interleukin 1 and tumor necrosis factor α in antibody-induced arthritis. J Exp Med 2002;196:77–85.

14. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171:6173–7.

15. Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc Natl Acad Sci U S A 2003;100:5986–90.

16. Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova FE, et al. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. Am J Pathol 2005;167:141–9.

17. Koenders MI, Kolls JK, Oppers-Walgreen B, van den Bersselaar L, Joosten LA, Schurr JR, et al. Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinase 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall–induced arthritis. Arthritis Rheum 2005;52:3239–47.

18. Chu CQ, Song Z, Mayton L, Wu B, Wooley PH. IFNγ deficient C57BL/6 (H-2^b) mice develop collagen induced arthritis with predominant usage of T cell receptor Vβ6 and Vβ8 in arthritic joints. Ann Rheum Dis 2003;62:983–90.

19. Manoury-Schwartz B, Chiocchia G, Bessis N, Abehsira-Amar O, Batteux F, Muller S, et al. High susceptibility to collagen-induced arthritis in mice lacking IFN-γ receptors. J Immunol 1997;158:5501–6.

20. Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P. Accelerated collagen-induced arthritis in IFN-γ receptor-deficient mice. J Immunol 1997;158:5507–13.

21. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis Rheum 2004;50:650–9.

22. Harrington JE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin-17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–32.

23. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang Y, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133–41.

24. Muraguchi A, Kishimoto T, Miki Y, Kuritani T, Kaieda T, Yoshizaki K, et al. T cell-replacing factor (TRF)-induced IgG secretion in human B blastoid cell line and demonstration of acceptors for TRF. J Immunol 1981;127:412–6.

25. Yoshizaki K, Nakagawa T, Kaieda T, Muraguchi A, Yamamura Y, Kishimoto T. Induction of proliferation and Igs-production in human B leukemic cells by anti-immunoglobulins and T cell factors. J Immunol 1982;128:1296–301.

26. Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti–interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum 2002;46:3143–50.

27. Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, et al. Treatment of rheumatoid arthritis with human-

ized anti–interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 2004;50:1761–9.

28. Schaller M, Burton DR, Ditzel HJ. Autoantibodies to GPI and creatine kinase in RA, and few human autoimmune sera detect GPI. Nat Immunol 2002;3:412–3.

29. Van Gaalen FA, Toes RE, Ditzel HJ, Schaller M, Breedveld FC, Verweij CL, et al. Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis. Arthritis Rheum 2004;50:395–9.

30. Kori Y, Matsumoto I, Zhang H, Yasukochi T, Hayashi T, Iwanami K, et al. Characterisation of Th1/Th2 type, glucose-6-phosphate isomerase reactive T cells in the generation of rheumatoid arthritis. Ann Rheum Dis 2006;65:968–9.

31. Maini R, St.Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, et al, for the ATTRACT Study Group. Infliximab (chimeric anti-tumour necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomized phase III trial. Lancet 1999;354:1932–9.

32. Lipsky PE, van der Heijde DM, St.Clair EW, Furst DE, Breedveld FC, Kalden JR, et al, for the Anti–Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med 2000;343:1594–602.

33. Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, et al. Treatment of rheumatoid arthritis with a recombinant human necrosis factor receptor (p75)-Fc fusion protein. N Engl J Med 1997;337:141–7.

34. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, et al. A trial of etanercept, a recombination tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999; 340:253–9.

35. Takagi N, Mihara M, Moriya Y, Nishimoto N, Yoshizaki K, Kishimoto T, et al. Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. Arthritis Rheum 1998;41:2117–21.

36. Joosten LA, Helsen MM, van de Loo FA, van den Berg WB. Anticytokine treatment of established type II collagen–induced arthritis in DBA/1 mice: a comparative study using anti-TNFα, anti–IL-1α/β, and IL-1Ra. Arthritis Rheum 1996;39:797–809.