Table 3

Synthetic peptides for screening T cell epitopes

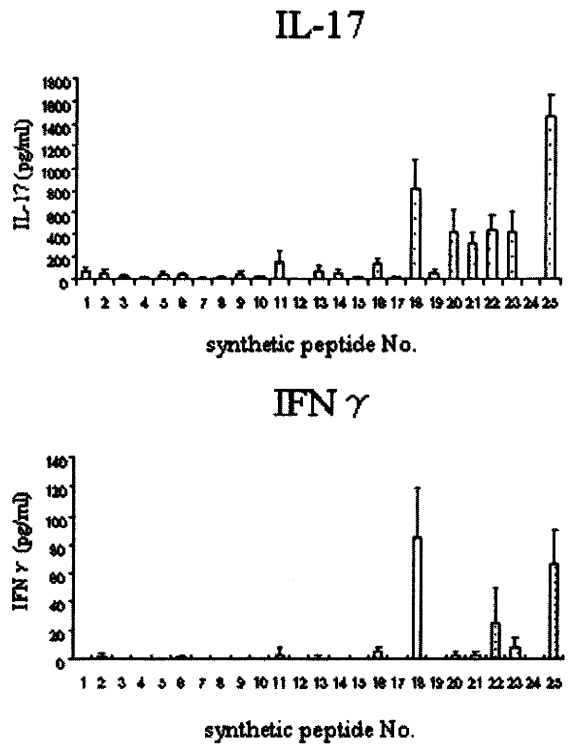
Peptide number	Peptide	Synthetic peptide sequence
1	1–20	H-MA**A**LTRDPQFQKLQQWYREH-OH
2	23–42	H-ELNLRR**L**FDANK**D**RFNHFSL-OH
3	37–56	H-FNHF**S**LTLNT**N**HG**H**ILVDYS-OH
4	51–70	H-ILVDY**S**KNL**V**TED**V**MRMLVD-OH
5	71–90	H-L**A**KSRGV**E**AARERMFNGEKI-OH
6	96–115	H-RAV**L**HVALRNRS**N**TPILVDG-OH
7	145–164	H-TGKTI**T**DV**I**NI**G**IGGSDLGP-OH
8	162–181	H-LGPLM**V**TEALK**P**YSSGGPRV-OH
9	168–187	H-TEALK**P**YSSGGPRVWYVSNI-OH
10	176–195	H-SGGPR**V**WYVSNIDGTHIAKT-OH
11	191–210	H-HIAKT**L**AQLNPESSLFIIAS-OH
12	200–219	H-N**P**ESSLFIIAS**K**TF**T**QETI-OH
13	225–244	H-AKEW**F**LQAAKD**P**SAVAKHFV-OH
14	238–257	H-AVAKH**F**VALSTN**T**TKVKEFG-OH
15	247–266	H-STNTTK**V**KEFGI**D**PQNMFEF-OH
16	280–299	H-IGLSI**A**LHVGFD**N**FEQLLSG-OH
17	313–332	H-EKNAPV**L**LALLGIWY**I**NCFG-OH
18	327–346	H-Y**I**NCFGCETHAMLPYDQYLH-OH
19	386–405	H-NGQHA**F**YQLIHQGTKMIPCD-OH
20	400–419	H-KMI**P**CD**F**LIPVQTQHPIRKG-OH
21	420–439	H-LHHKI**L**LA**N**FLAQTEALMRG-OH
22	445–464	H-ARKELQA**A**GKS**P**EDLERLLP-OH
23	484–503	H-PFMLG**A**LVAMY**E**HKIFVQGI-OH
24	533–552	H-AQVT**S**HDAST**N**GLI**N**FIKQQ-OH
25	539–558	H-DASTNG**L**I**N**FIKQQREARVQ-OH

Listed are 25 20-mer unpurified peptides in which each core sequence were centred around. Amino acid residues constituting the core sequence and those thought to bind anchors of I-A^q are underlined and shown in bold letters, respectively.

acids homology to $mGPI_{544-558}$ (GLISFIKQQRDTKLE). The draining lymph node cells from mice immunised with $hGPI_{325-339}$ or $hGPI_{544-558}$were cultured in the presence of $hGPI_{325-339}$, $mGPI_{325-339}$, $hGPI_{544-558}$ or $mGPI_{544-558}$ for 24 hours. The $hGPI_{325-339}$-primed cells had distinct cross-reactive immune reaction to $mGPI_{325-339}$ by producing IL-17, whereas the $hGPI_{544-558}$ primed cells did not cross-react to $mGPI_{544-558}$ (Figure 5a). As compared with the draining lymph node cells of $hGPI_{325-339}$-immunised mice, IL-17 production was not remarkable in that of $hGPI_{544-558}$-immunised mice even when the corresponding peptide was used as an antigen for *in vitro* stimulation (Figure 5a). The production of IFN-γ was much lower than that of IL-17, and IL-4 production was not detectable independent of immunisation patterns and antigens for *in vitro* stimulation (data not shown).

It has been reported that Th17 cells are not the only cellular sources of IL-17, but $CD8^+$ T cells, natural killer T cells and γδT cells are also capable of producing IL-17 [17-22]. Therefore, we investigated the IL-17 producing cells using flow cytometry. The draining lymph node cells from mice immunised with $hGPI_{325-339}$ or $hGPI_{544-558}$ were stimulated with $hGPI_{325-339}$ and $mGPI_{325-339}$, or $hGPI_{544-558}$ and $mGPI_{544-558}$, respectively. Intracellular cytokine staining was performed without nonspecific stimulants, such as phorbol myristate acetate or ionomycin. We confirmed that immunisation of $hGPI_{325-339}$ induced antigen-specific Th17 cells, which cross-reacted with $mGPI_{325-339}$. However, immunisation of $hGPI_{544-558}$ induced neither $hGPI_{544-558}$-specific Th17 cells nor Th17 cells that can cross-react with $mGPI_{544-558}$ remarkably (Figure 5b). These data indicate that induction of antigen-specific Th17 cells and

Figure 1



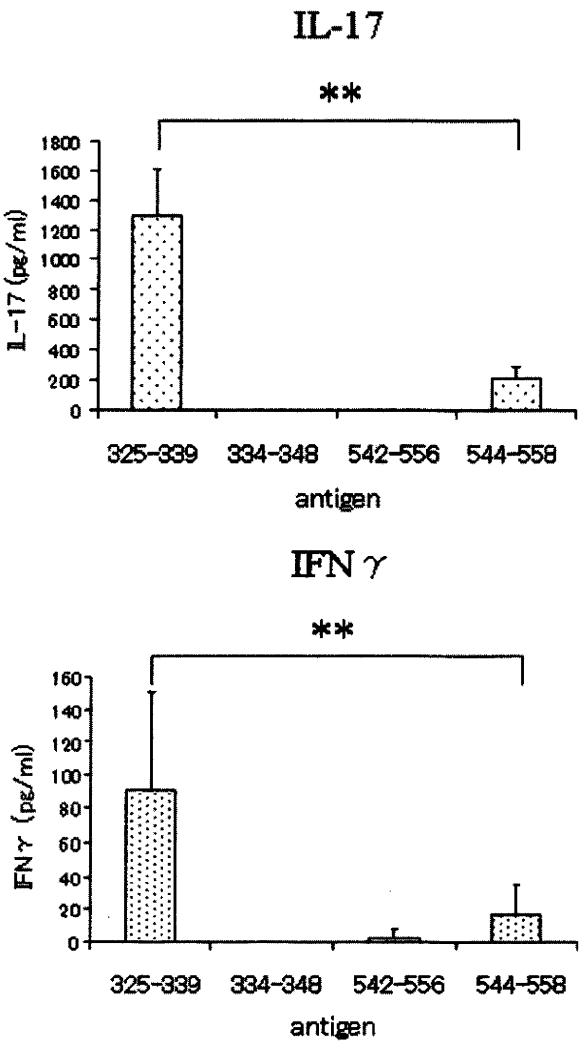
Synthetic peptides number 18 and 25 produced marked simulation of glucose-6-phosphate isomerase (GPI) primed CD4+ T cells. Mice were sacrificed on day 7 after immunisation. CD4+ T cells were purified from spleen cells of GPI-immunised DBA/1 mice. GPI-primed CD4+ T cells and antigen presenting cells (APCs) were co-cultured with 10 μM of synthetic peptide for 24 hours. The supernatants were assayed for interferon (IFN) γ and interleukin (IL) 17 by ELISA. Data are averages ± standard deviation of three culture wells. Representative data of three independent experiments.

cross-reactivity with mouse GPI might be the pathogenesis of peptide-induced arthritis.

Immunisation of human $GPI_{325-339}$ leads B cells to produce anti-mouse GPI antibodies

To explore the importance of autoantibodies, we measured anti-human GPI antibodies and anti-mouse GPI antibodies in mice immunised with $hGPI_{325-339}$, $hGPI_{544-558}$ and $hGPI_{325-339}$ plus $hGPI_{544-558}$ by ELISA. Mice immunised with rhGPI and the two peptides ($hGPI_{325-339}$ plus $hGPI_{544-558}$) produced high titres of anti-human GPI antibodies and anti-mouse GPI antibodies, and mice immunised with $hGPI_{325-339}$ and $hGPI_{544-558}$ hardly produced any anti-human GPI antibodies. However, mice immunised with $hGPI_{325-339}$ produced significantly higher titres of anti-mouse GPI antibodies than mice immunised with $hGPI_{544-558}$ (Figure 6a). It is noteworthy that immunisation with the two peptides ($hGPI_{325-339}$ plus $hGPI_{544-558}$) induced significantly higher titres of anti-mouse

Figure 2



$GPI_{325-339}$ is a major epitope. Mice were sacrificed on day 7 after immunisation. CD4+ T cells were purified from splenocytes of glucose-6-phosphate isomerase (GPI) immunised DBA/1 mice. GPI-primed CD4+ T cells and antigen presenting cells (APCs) were co-cultured with 10 μM of synthetic peptide $hGPI_{325-339}$, $hGPI_{334-348}$, $hGPI_{542-556}$ or $hGPI_{544-558}$ for 24 hours. The purity of each peptide was 90%. The supernatants were assayed for interferon (IFN) γ and interleukin (IL) 17 by ELISA. Data are averages ± standard deviation of five culture-wells. **$p < 0.01$ (Mann-Whitney's U test). Representative data of three independent experiments.

GPI antibodies than that with $hGPI_{325-339}$ alone, whereas the severity and incidence of arthritis in mice immunised with two peptides ($hGPI_{325-339}$ plus $hGPI_{544-558}$) were comparable with those in mice immunised with $hGPI_{325-339}$ alone (Figures 3a and 6a).

Table 4

Re-synthesised peptides used for determining a major epitope

Peptide number	Peptide	Synthetic peptide sequence
18	327–346	H-YINCFGCETHAMLPYDQYLH-OH
	325–339	H-IWYINCFGCETHAML-OH
	334–348	H-ETHAMLPYDQYLHRF-OH
25	539–558	H-DASTNGLINFIKQQREARVQ-OH
	542–556	H-TNGLINFIKQQREAR-OH
	544–558	H-GLINFIKQQREARVQ-OH

The 15-mer peptides were synthesised with 90% purity, containing each core sequence of number 18 peptide ($GPI_{327-346}$) and number 25 peptide ($GPI_{539-558}$). Amino acid residues constituting the core sequence and those thought to bind the anchors of I-A^q are underlined and shown in bold letters, respectively.

We further investigated the difference of the correlation between anti-mouse GPI antibodies and arthritis score among immunisation patterns. Each of the three different immunisation patterns (rhGPI, $hGPI_{325-339}$ and $hGPI_{325-339}$ plus $hGPI_{544-558}$) showed no positive correlation between anti-mouse GPI antibodies and arthritis score (Table 5).

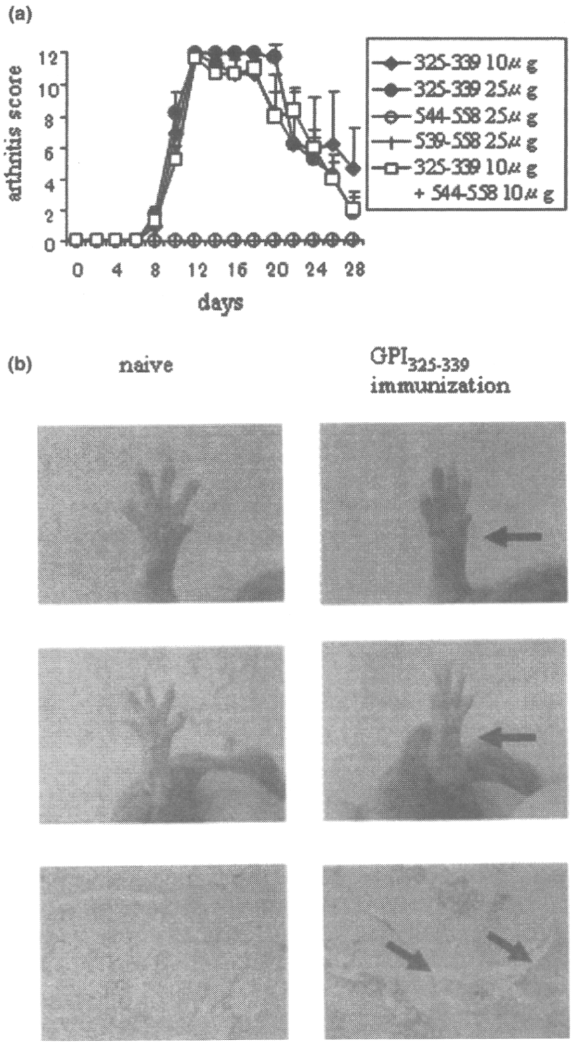
Next, we investigated the existence of IgG on the cartilage surface by immunohistology, because GPI were proved to deposit on the cartilage surface of normal naïve mice [23]. The cryostat sections of ankle joints from naïve mice and mice immunised with $hGPI_{544-558}$ did not show IgG deposit on the cartilage surface. However, those from mice immunised with rhGPI and $hGPI_{325-339}$ showed IgG deposits (Figure 6b). These data indicate that anti-mouse GPI antibodies may play a role in the development of peptide-induced arthritis.

Discussion

GPI, a ubiquitous glycolytic enzyme, is a new autoantigen candidate in autoimmune arthritis [5,6]. GPI-induced arthritis is induced by immunisation of genetically unaltered DBA/1 mice with rhGPI [1]. We report here the therapeutic efficacies of mAb to tumour necrosis factor-α and IL-6 and CTLA-4 Ig in this model [3]. Moreover, $CD4^+$ T cells, especially Th17 cells, seem to be more important than B cells, because administration of anti-CD4 mAb or anti-IL-17 mAb markedly ameliorate the progress of arthritis independent of anti-GPI antibodies titres [1,2]. Therefore, exploring the epitope of $CD4^+$ T cells and its arthritogenic effect is important for understanding the pathological mechanisms.

In this study, we investigated the binding motif of I-A^q from T cell epitopes considered to bind to I-A^q, synthesised peptides of epitope candidates and identified $hGPI_{325-339}$ as a major epitope. Interestingly, the MHC binding residues of $hGPI_{325-339}$(IWYINCFGCETHAML) at P1, P4 and P7 were the same as those for bovine $CII_{256-270}$ (GEP-

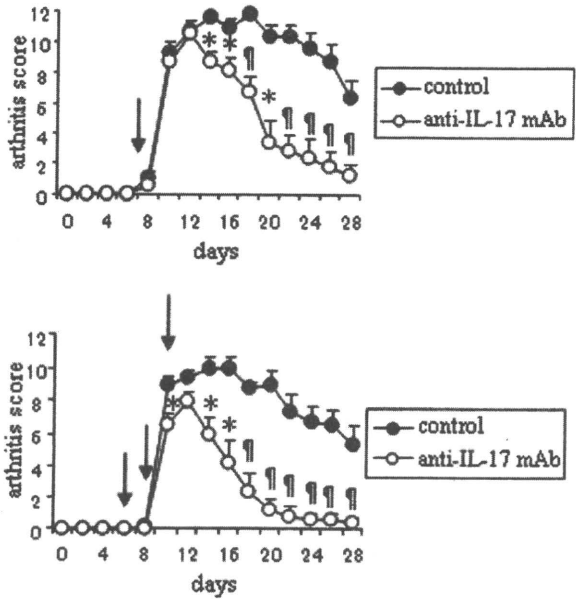
Figure 3



Immunisation with $hGPI_{325-339}$ induces severe polyarthritis. DBA/1 mice were immunised with 25 μg of $hGPI_{325-339}$, $hGPI_{539-558}$ or $hGPI_{544-558}$, or 10 μg each of $hGPI_{325-339}$ plus $hGPI_{544-558}$, and 200 ng of pertussis toxin was injected intraperitoneally on days 0 and 2 after immunisation. (a) The mean arthritis score (± standard error of the mean (SEM)) of five mice in one representative experiment of two independent experiments. (b) Severe swelling of the wrist (upper panels) and ankle joints (middle panels) in mice immunised with 25 μg of $hGPI_{325-339}$ compared with naïve mice (arrowheads). Histological analysis of haematoxylin & eosin-stained sections of ankle joints taken from naïve mice and mice on day 14 after $hGPI_{325-339}$ immunization (lower panels) showed severe synovitis with massive infiltration of cells and hyperplasia of synovial tissue (arrowheads).

induced arthritis [4]. These findings indicate that the binding motif (P1 I, P4 F, P7 E) might have high binding affinity with I-A^q, and the peptides with this motif-MHC complexes might be effectively recognised by TCRs and could be arthritogenic in some condition. Although immunisation with a fragment of

Figure 4



Anti-IL-17 monoclonal antibody (mAb) suppresses the development of arthritis. DBA/1 mice were immunised with 25 μg of $hGPI_{325-339}$, and 200 ng of pertussis toxin was injected intraperitoneally on days 0 and 2 after immunisation. 100 μg of anti-IL-17 mAb or isotype control (control) was administered intraperitoneally on day 7 (upper panel) or day 6, 8, and 10 (lower panel) after immunisation (arrow). Mean arthritis score (± standard error of the mean (SEM)) of five mice per group. Representative data of two independent experiments. * $p < 0.05$, ¶ $p < 0.01$ (Mann-Whitney's U test).

cyanogen bromide of bovine CII, CB11 ($CII_{124-402}$), which contains the dominant epitope, can induce arthritis, the severity and incidence are much lower than arthritis induced by bovine CII protein [4]. Other fragments (CB8, CB9, CB10 and CB12) do not induce arthritis, as is explained by the production of anti-bovine CII antibodies. Immunisation with CB11 fragment produces five times more antibodies to bovine CII than any other fragment [4]. The observation that administration of anti-CD4 mAb after the onset of arthritis did not ameliorate the arthritis [24,25] and a combination of mAb to CII can passively transfer arthritis to naïve mice [26] also emphasises the importance of autoantibodies to the induction of collagen-induced arthritis.

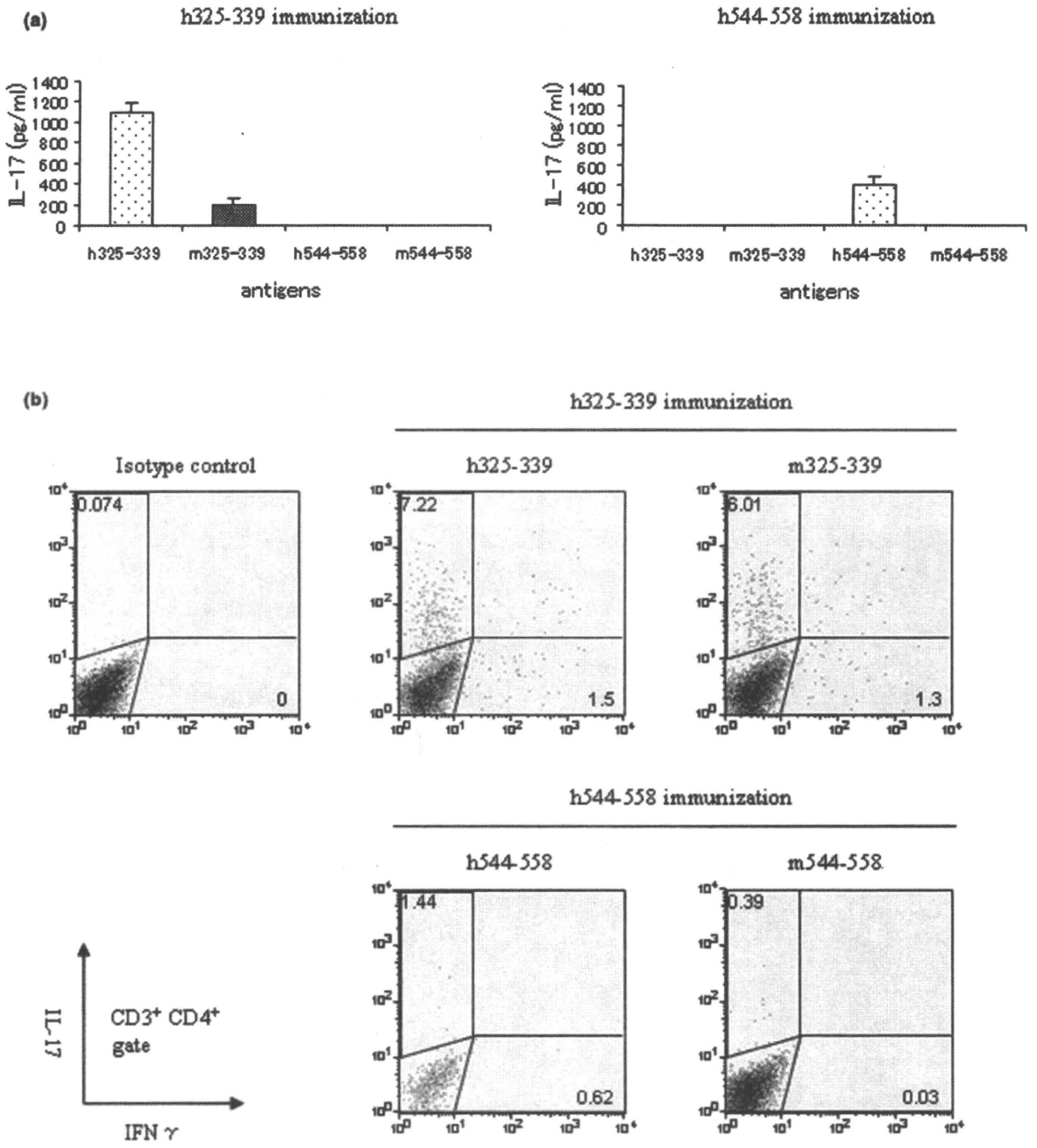
Our study demonstrated that immunisation with $hGPI_{325-339}$ induced antigen-specific Th17 cells, which can cross-react with $mGPI_{325-339}$ and lead B cells to produce anti-mouse GPI antibodies. However, immunisation with $hGPI_{544-558}$ could not even induce $hGPI_{544-558}$-specific Th17 cells. The difference of ability of Th17 induction between two peptides may come from MHC-binding affinity and TCR-binding affinity. A peptide that is likely to bind to MHC class II with high affinity and interacts strongly with the T cell receptor tends to stimulate Th1-cell response, whereas a peptide with low binding affinity to MHC class II and T cell receptor tends to elicit Th2-cell response [27,28]. Although the relationship between Th17 differentiation and the strength of TCR signalling and MHC-binding affinity has not been clarified, it is possible that the difference in amino acid sequences between $hGPI_{325-339}$ and $hGPI_{544-558}$ might affect the I-Aq binding affinity and the TCR signalling, and consequently lead to the difference in extent of antigen-specific Th17 cells. In this study, we did not detect any IL-4 production, which is an adjuvant effect of *Mycobacterim tuberculosis* and pertussis toxin.

In K/BxN mice expressing $I\text{-}A^{g7}$ as MHC class II molecules, $mGPI_{282-294}$-specific $CD4^+$ T cells lead B cells to produce anti-mouse GPI antibodies [16]. The anti-mouse GPI antibodies from K/BxN mice have such high affinity that IgG transfer of K/BxN mice can provoke arthritis in normal mice [6]. In comparison, the anti-mouse GPI antibodies from GPI-induced arthritis alone are not sufficient for the development of arthritis because IgG transfer from mice immunised with rhGPI can not provoke arthritis. However, IgG signalling through FcγR seems necessary for the induction of GPI-induced arthritis because FcγR-deficient mice are resistant to arthritis [1]. Moreover, the data that transfer of rhGPI-primed or $hGPI_{325-339}$-primed Th17 cells to naïve DBA/1 mice can not induce arthritis emphasises the necessity of anti-mouse GPI antibodies (unpublished observation). Considering the data that there are no positive correlation between anti-mouse GPI antibodies and arthritis score [[29] and unpublished observation], and arthritis-resistant mice like C57BL/6 produce as high titres of anti-mouse GPI antibodies as DBA/1 when immunised with rhGPI (1 and unpublished observation), anti-mouse GPI antibodies may play a subordinate role in the development of GPI-induced arthritis and peptide-induced arthritis in DBA/1 mice.

In the process of epitope screening, the response to $hGPI_{539-558}$ peptide was comparable with that to $hGPI_{327-346}$ peptide; however, the response to $hGPI_{542-556}$ and $hGPI_{544-558}$, which were synthesised with 90% purity, was lower than that to $hGPI_{539-558}$ peptide. Furthermore, the response to $hGPI_{539-558}$, which was re-synthesised with 90% purity, was much lower than to $hGPI_{325-339}$ or to $hGPI_{539-558}$ peptide for screening (data not shown). These results could be explained by differences in the purity of the synthetic peptides. The synthetic peptides used for screening (peptides numbers 1 to 25, Table 2) were unpurified, and the purity of each peptide would have been quite different, although the exact purity was unchecked by the product maker. Therefore, it is possible that the purity of number 25 peptide might have been much higher than that of number 18 peptide, or alternatively, number 25 peptide may have contained other peptides through peptide synthesis.

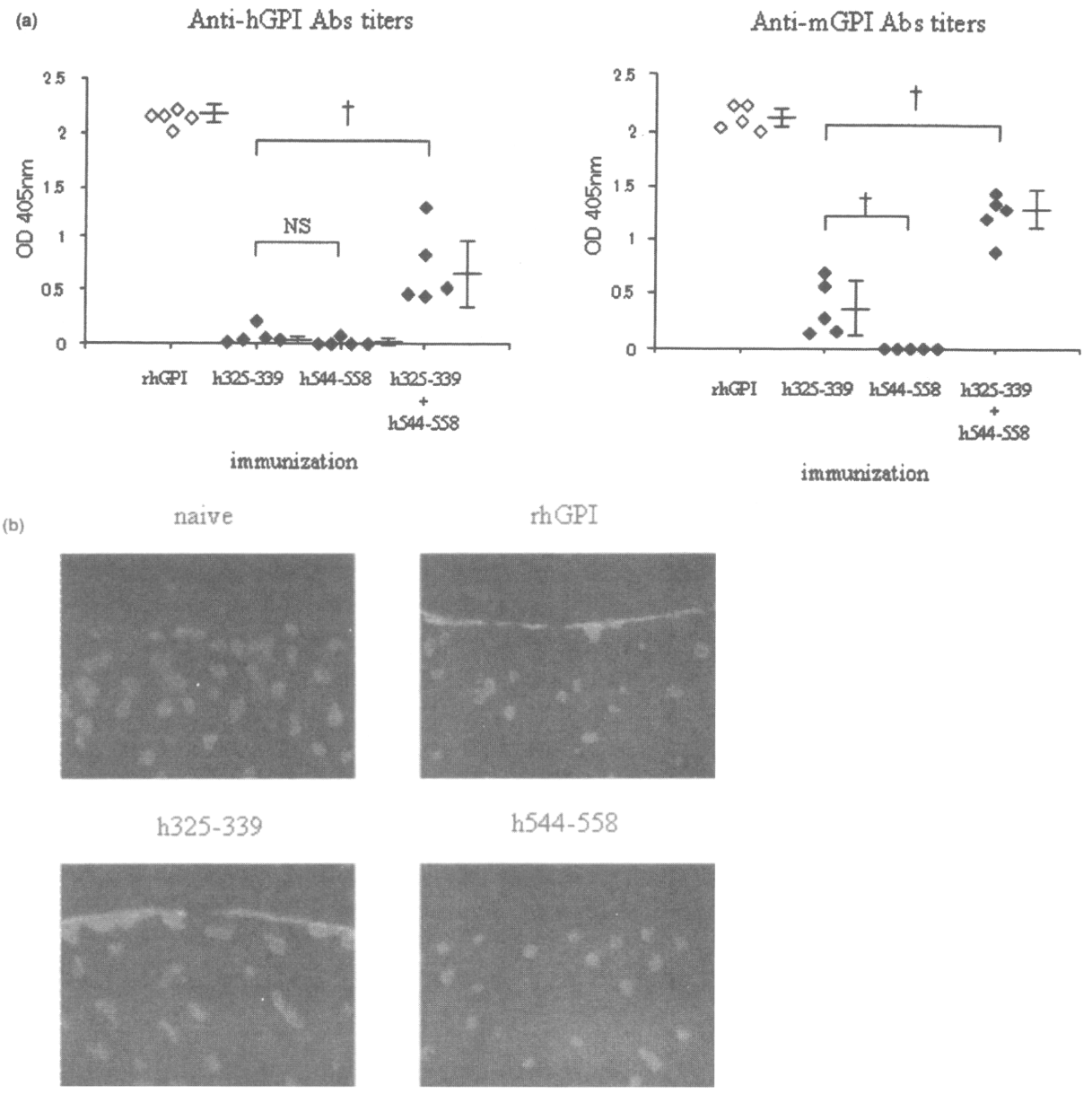
From a probability point of view, it is possible that other epitopes exist in some regions of human GPI-amino acid

Figure 5



Cross-reactivity with peptides derived from mouse glucose-6-phosphate isomerase (GPI). (a) Draining lymph node (DLN) cells taken from $hGPI_{325-339}$-immunised mice on day 5 were cultured with 10 μM of $hGPI_{325-339}$, $mGPI_{325-339}$, $hGPI_{544-558}$ or $mGPI_{544-558}$ for 24 hours. The supernatants were assayed for interleukin (IL) 17 by ELISA. Data are averages ± standard deviation of three culture-wells. Representative data of three independent experiments. (b) DLN cells taken from $hGPI_{325-339}$- or $hGPI_{544-558}$-immunised mice on day 5 were cultured with 10 μM of $hGPI_{325-339}$ and $mGPI_{325-339}$ or $hGPI_{544-558}$ and $mGPI_{544-558}$, respectively. GoldiStop was added at the last four hours of each culture. Flow cytometry for IL-17 and interferon (IFN) γ was gated in $CD3^+$, $CD4^{high}$ cells. Representative flow cytometry data of three independent experiments with two mice per experiment.

Figure 6



Titres of anti-mouse glucose-6-phosphate isomerase (GPI) antibodies were elevated in mice with arthritis. (a) Sera were taken on day 14 from mice immunised with recombinant human (rh) GPI, $hGPI_{325-339}$, $hGPI_{544-558}$ or $hGPI_{325-339}$ plus $hGPI_{544-558}$, and the titres of anti-human GPI antibodies and anti-mouse GPI antibodies were analysed by ELISA. Each symbol represents a single mouse. Data are mean optimal density ± standard deviation. †$p < 0.01$ (Mann-Whitney's U test). Representative data of two independent experiments. (b) Ankle joints were taken on day 14 from mice immunised with rhGPI, $hGPI_{325-339}$ or $hGPI_{544-558}$. Cryostat sections of ankle joints were stained with anti-mouse IgG (red), and nuclei were counterstained with 4',6-diamidino-2-phenyindole dilactate (blue). Representative data of three independent experiments.

sequence from which we did not synthesise the peptides, because I-A^q may have another binding motif and our synthesised peptides covered only the 399/558 (71.5%) amino acid residues of human GPI protein, not the whole length. However, two experimental pieces of data support that $hGPI_{325-339}$ may be the dominant epitope. One is that immunisation with $hGPI_{325-339}$ provoked arthritis similar to that induced by rhGPI protein. The other is that intraperitoneal injection of $hGPI_{325-339}$ after the onset of arthritis significantly ameliorated the progress of arthritis (data not shown). Because systemic

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 $I\text{-}A^q$ 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

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Research article

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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

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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_4Cl (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 IgG_1 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 $MgCl_2$ [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

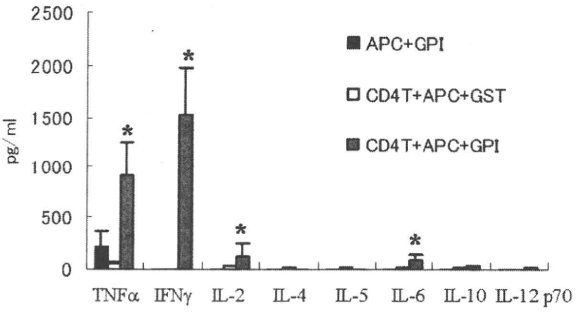
All data were expressed as mean ± standard error of the mean. Differences between groups were examined for statistical significance by using Mann-Whitney's U test. $P < 0.05$ denoted the presence of a statistically significant difference.

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 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).

Figure 1



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 (1×10^6 cells/ml) were stimulated with 5 μg/ml GPI (or glutathione *S*-transferase [GST]) and antigen-presenting cells (APCs; 2×10^5 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.

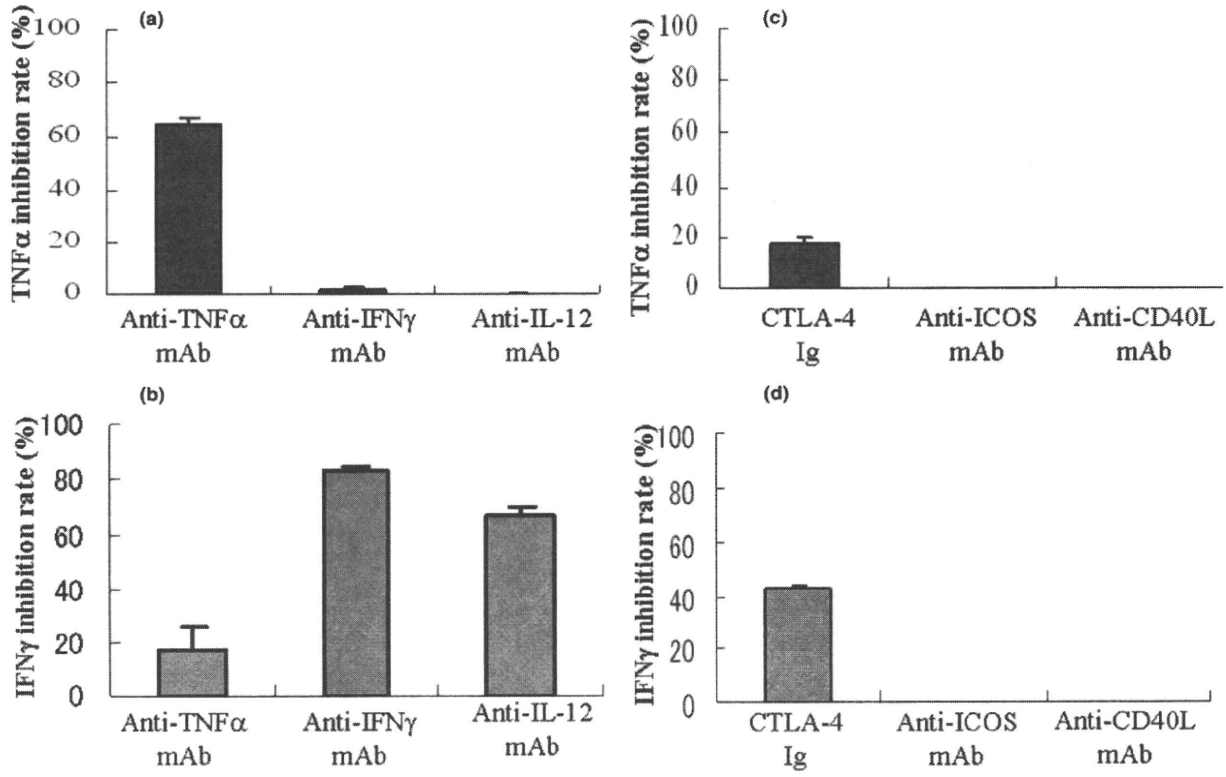
GPI induces production of TNF-α and IFN-γ by spleen cells at onset of arthritis

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.

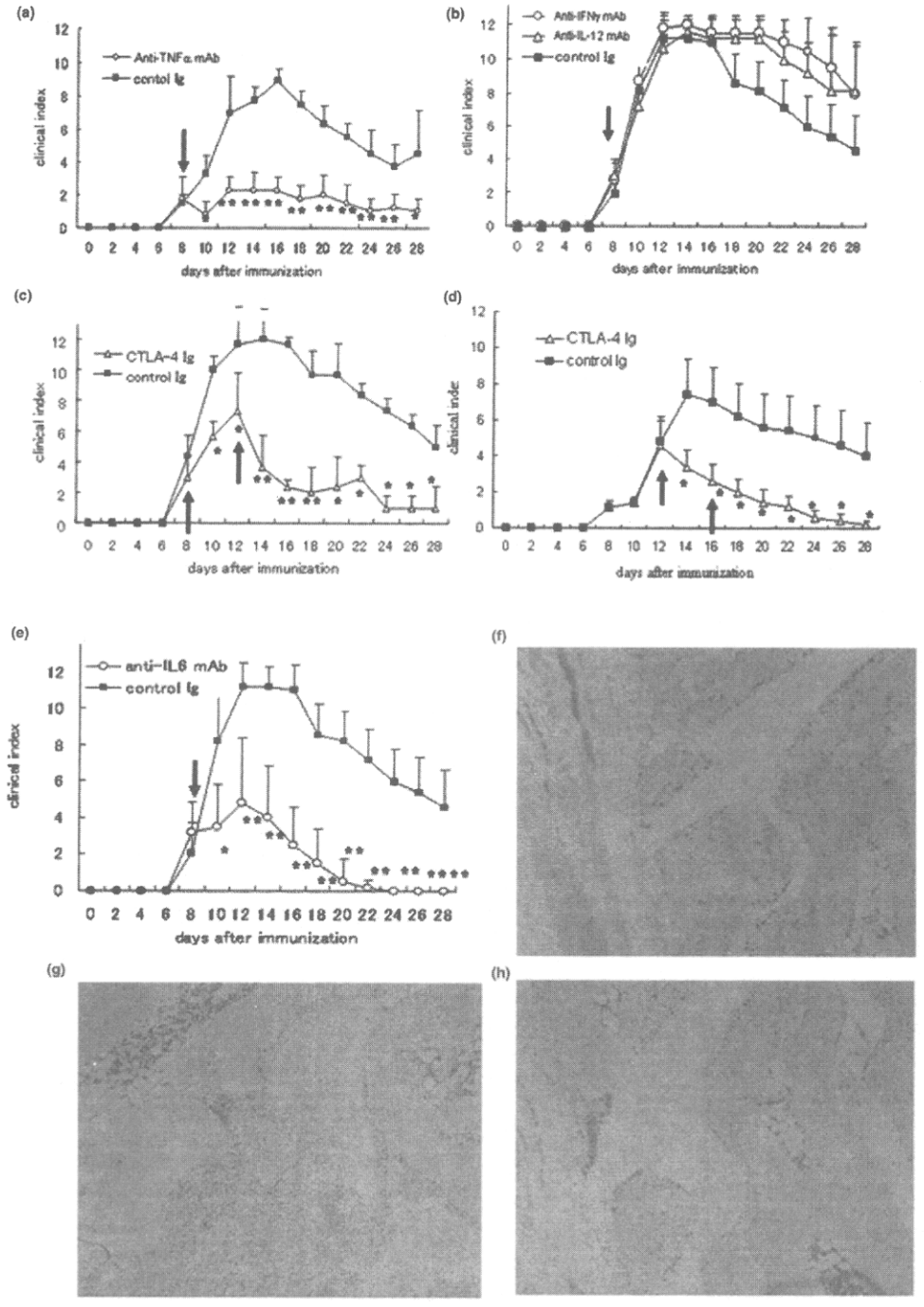
2.7%; Figure 2a), but not by anti-IL-12 mAb (0%; Figure 2a). On the other hand, IFN-γ production was inhibited by anti-IFN-γ mAb (82.5 ± 1.2%; Figure 2b) as well as by anti IL-12 mAb (67.5 ± 2.5%; Figure 2b), and weakly by anti-TNF-α mAb (17.2 ± 9.2%; Figure 2b). These results suggest that TNF-α production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

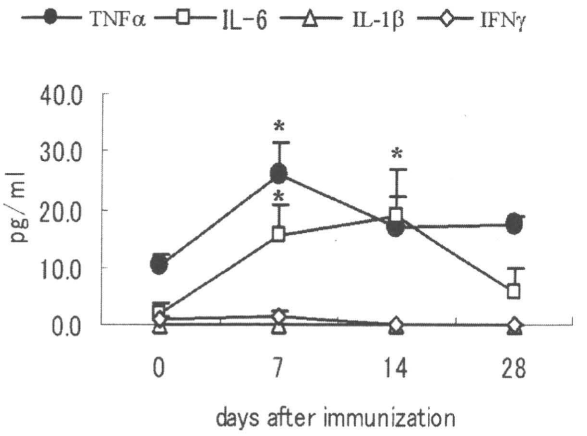
To investigate the effect of CTLA-4Ig *in vivo*, we treated arthritic mice with CTLA-4Ig on days 8 and 12, or on days 12 and 14. A marked improvement was seen after the second treatment (on days 8 and 12), probably because of a reduction in effector T cells at that stage (Figure 4c, and hematoxylin and 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.

Figure 4

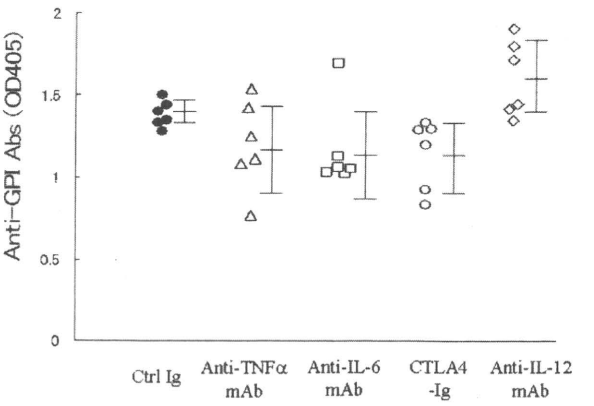


Concentration of inflammatory cytokines in serum of mice with GPI-induced arthritis. After immunization with glucose-6-phosphate isomerase (GPI), serum samples were collected from GPI-induced DBA/1 mice at the indicated time points (days 0, 7, 14, and 28). Serum concentrations of tumor necrosis factor (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.

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 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 ($H\text{-}2^b$) [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.

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

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

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

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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 m*M* IPTG to the medium, which was followed by a further culture overnight at 30°C. The bacteria were lysed with a sonicator, and the supernatant was purified with a glutathione–Sepharose column (Pharmacia). The purity was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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

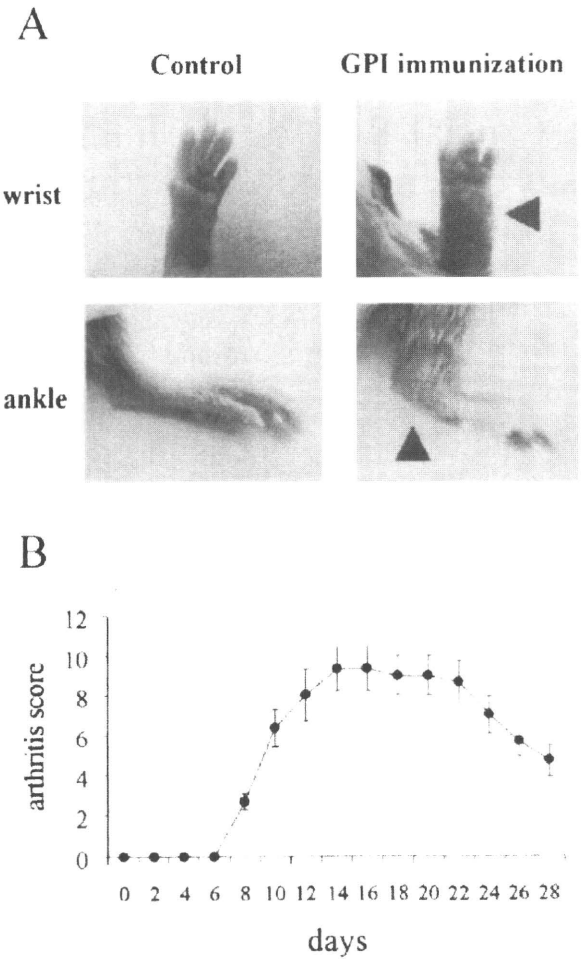

Figure 1. Induction of severe polyarthritis by immunization with recombinant human glucose-6-phosphate isomerase (GPI). DBA/1 mice were immunized with 300 μg of recombinant human GPI, and the development of arthritis was monitored visually and scored on a scale of 0–3 (see Materials and Methods for details).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

Analysis of cytokine profiles. Mice were killed on day 7 or day 14. Spleens were harvested and hemolyzed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$, and 0.004% disodium EDTA in phosphate buffered saline (PBS). Single-cell suspensions were prepared in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin,

and 50 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 μg/ml of mitomycin C were used as antigen-presenting cells (APCs). Purified CD4+ T cells and APCs were cocultured with 5 μg/ml of GPI at a ratio of 5:1 for 24 hours at 37°C in an atmosphere containing 5% CO_2. The supernatants were assayed for interferon-γ (IFNγ), IL-4, and IL-17 by enzyme-linked immunosorbent assay (ELISA) using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

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

Anti-GPI antibody analysis. Sera were obtained on day 28 or day 35 and diluted 1:500 in blocking solution (25% Block-Ace [Dainippon Sumitomo Pharma, Osaka, Japan] in PBS) for analysis of antibody. Then, 96-well plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 5 μg/ml of recombinant human GPI for 12 hours at 4°C. After washing twice with washing buffer (0.05% Tween 20 in PBS), the blocking solution was applied for 2 hours at room temperature to block nonspecific binding. After 2 washes, 150 μl of diluted sera was added, and the plates were incubated for 2 hours at room temperature. After 3 washes, alkaline phosphatase (AP)–conjugated anti-mouse IgG was added at a final dilution of 1:5,000 for 1 hour at room temperature. After 3 washes, color was developed with substrate solution, consisting of 1 tablet of AP tablet (Sigma-Aldrich) per 5 ml of AP reaction solution (9.6% diethanolamine and 0.25 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 (1×10^6/ml) were stimulated with 100 μg/ml of recombinant human GPI in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) for 24 hours. 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 (2×10^7/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 μg/ml of recombinant human GPI at 1×10^6/ml in 96-well round-bottomed plates (Nunc) for 60 hours and then analyzed by flow cytometry.

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

RESULTS

Induction of severe symmetric polyarthritis by immunization with GPI. For the induction of arthritis, we immunized DBA/1 mice with 300 μg of recombinant human GPI emulsified with Freund's complete adjuvant. Of the 177 mice immunized with recombinant human GPI, 167 (94.4%) developed severe swelling of the wrist and ankle joints (Figure 1A). The arthritis appeared on

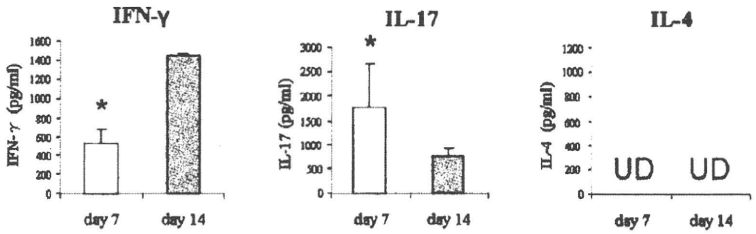

Figure 2. Differentiation of glucose-6-phosphate isomerase (GPI)–specific CD4+ T cells into Th1 and Th17 cells. CD4+ T cells and mitomycin C–treated antigen-presenting cells were stimulated for 24 hours with GPI on either day 7 (induction phase) or day 14 (effector phase) and then assessed for the production 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).

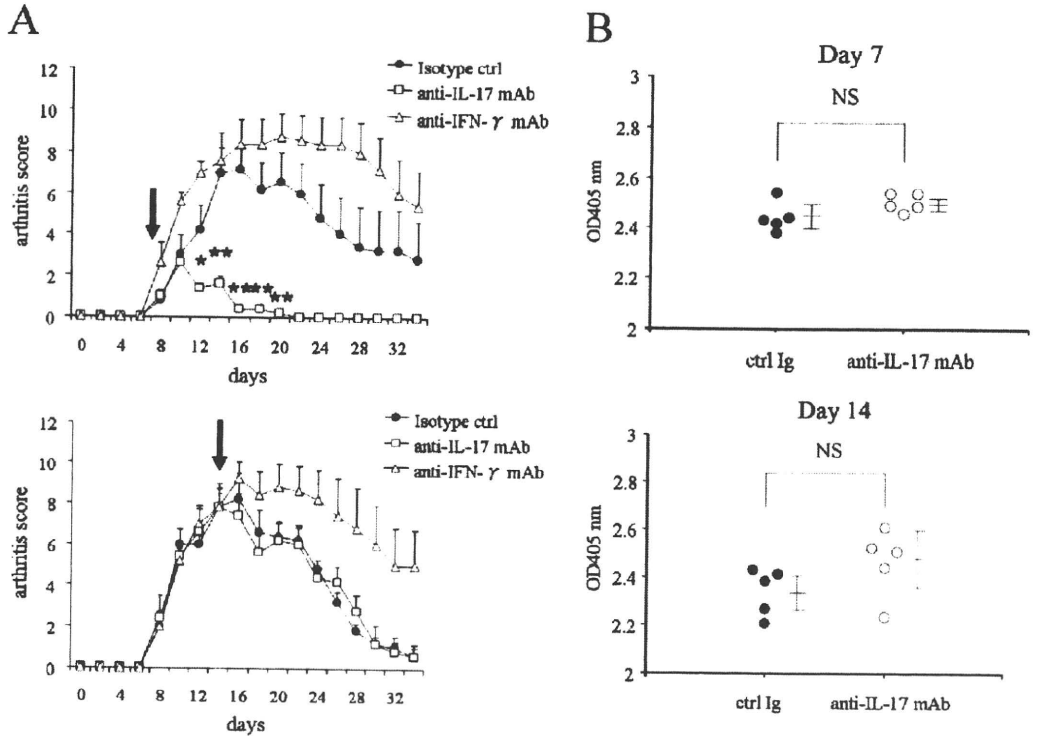

Figure 3. Suppression of the development of glucose-6-phosphate isomerase (GPI)–induced arthritis by treatment with anti–interleukin-17 (anti–IL-17) monoclonal antibody (mAb). **A,** Arthritis scores following intraperitoneal injection of 100 μg of anti–IL-17 mAb or anti–interferon-γ (anti-IFNγ) mAb 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).