panel). Moreover, in lymph nodes, TIARP mRNA was upregulated at day 28. But the expression of TIARP mRNA in lymph nodes was very weak compared with the other tissues (Figure 2c, bottom panel). We also confirmed that the mRNA expression of TIARP in joints was upregulated at day 28, but not at day 14, in mice with collagen-induced arthritis and that expression correlated with joint swelling (data not shown). These findings suggest that the systemic upregulation of TNFα and TIARP is involved in the early phase of the disease and that TIARP expression in arthritic joints seems to correlate with joint swelling.

Treatment with anti-tumor necrosis factor-alpha monoclonal antibody suppresses TIARP expression

To test the therapeutic efficacy of anti-TNFα mAb, we injected anti-TNFα mAb after clinical onset of arthritis at day 8. A single injection of 100 μg of anti-TNFα mAb at day 8 ameliorated the disease, as indicated by a rapid fall in the semiquantitative score of arthritis (Figure 3a) [3]. To explore the relevance of the therapeutic effect of anti-TNFα mAb on TIARP expression, we evaluated TIARP expression after injection of anti-TNFα mAb in mice with GPI-induced arthritis. Treatment of mice with anti-TNFα mAb resulted in downregulation of TIARP expression in spleen relative to control Ig injection, although no treatment-related change in TIARP expression was noted at day 14 ($P = 0.03$) (Figure 3b, top panel). However, in joints, expression of TIARP mRNA was almost comparable between the treatment with anti-TNFα mAb and control Ig. These results suggest that TNF antagonism induces TIARP downregulation and results in the amelioration of arthritis.

$CD11b^+$ cells are the main source of TIARP mRNA in splenocytes of arthritic mice

In the next set of experiments, splenocytes of arthritic mice were separated into $CD4^+$, $CD19^+$, $CD11b^+$, and $CD11c^+$ cells by MACS. In naïve mice, $CD19^+$, $CD11b^+$, and $CD11c^+$ cells expressed TIARP, and induction of arthritis was associated with upregulation of TIARP mRNA in $CD11b^+$ cells, as demonstrated by quantitative PCR ($P < 0.05$ at day 7) (Figure 4a). These findings suggest the induction of TIARP in $CD11b^+$ cells in splenocytes of arthritic mice, especially during the early phase of the disease.

Localization of TIARP protein in proliferative synovium

Next, immunohistochemical analysis was conducted to determine the distribution of TIARP in the arthritic joints. For this purpose, we generated polyclonal anti-TIARP antibodies using rats, as described previously [5]. TIARP protein was clearly identified in the proliferative synovium of arthritic joints of mice (at day 14) (Figure 4b, top panels), whereas almost no signal was detected in naïve mice (Figure 4b, bottom panels). While these findings indicate TIARP protein expression in the synovium, the results do not link such expression with an ameliorative or damaging effect on the synovium.

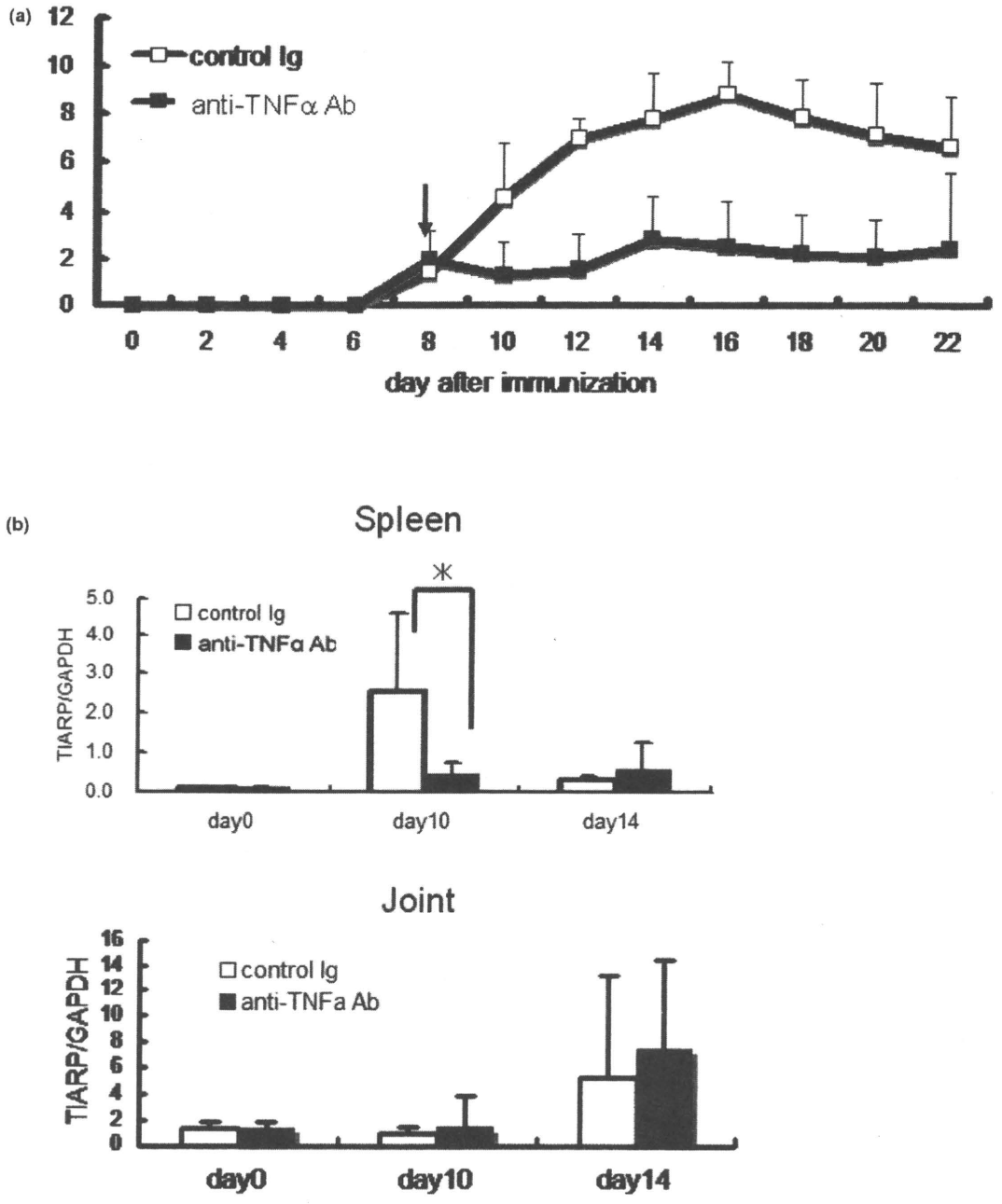
Overexpression of STEAP4 in joints of rheumatoid arthritis patients and its localization in $CD68^+$ cells

To determine the role of STEAP4 (the human ortholog of mouse TIARP)in human RA, we analyzed PBMCs from RA patients and healthy subjects and synovia from RA patients. For comparison, we also screened other STEAP family members such as STEAP2 and STEAP3 using the same method. For PBMCs, STEAP4 mRNA was detected in only one RA patient (1/3). Importantly, STEAP4 mRNA was highly expressed in all four RA synovia whereas only faint bands were noted for other STEAP families (Figure 5a). Next, using several numbers of synovial tissues from patients with RA and OA, we investigated the expression of STEAP4 mRNA in synovium of patients with RA and OA. Relative expression of STEAP4 was almost comparable between RA and OA, although expression variation tended to be enhanced in RA synovium (Figure 5b). Moreover, immunohistochemical analysis of synovia of RA patients showed co-localization of STEAP4 protein with CD68, a marker for human macrophages (Figure 5c). These findings suggest that STEAP4 is specifically expressed in joints and is localized with $CD68^+$ cells.

Discussion

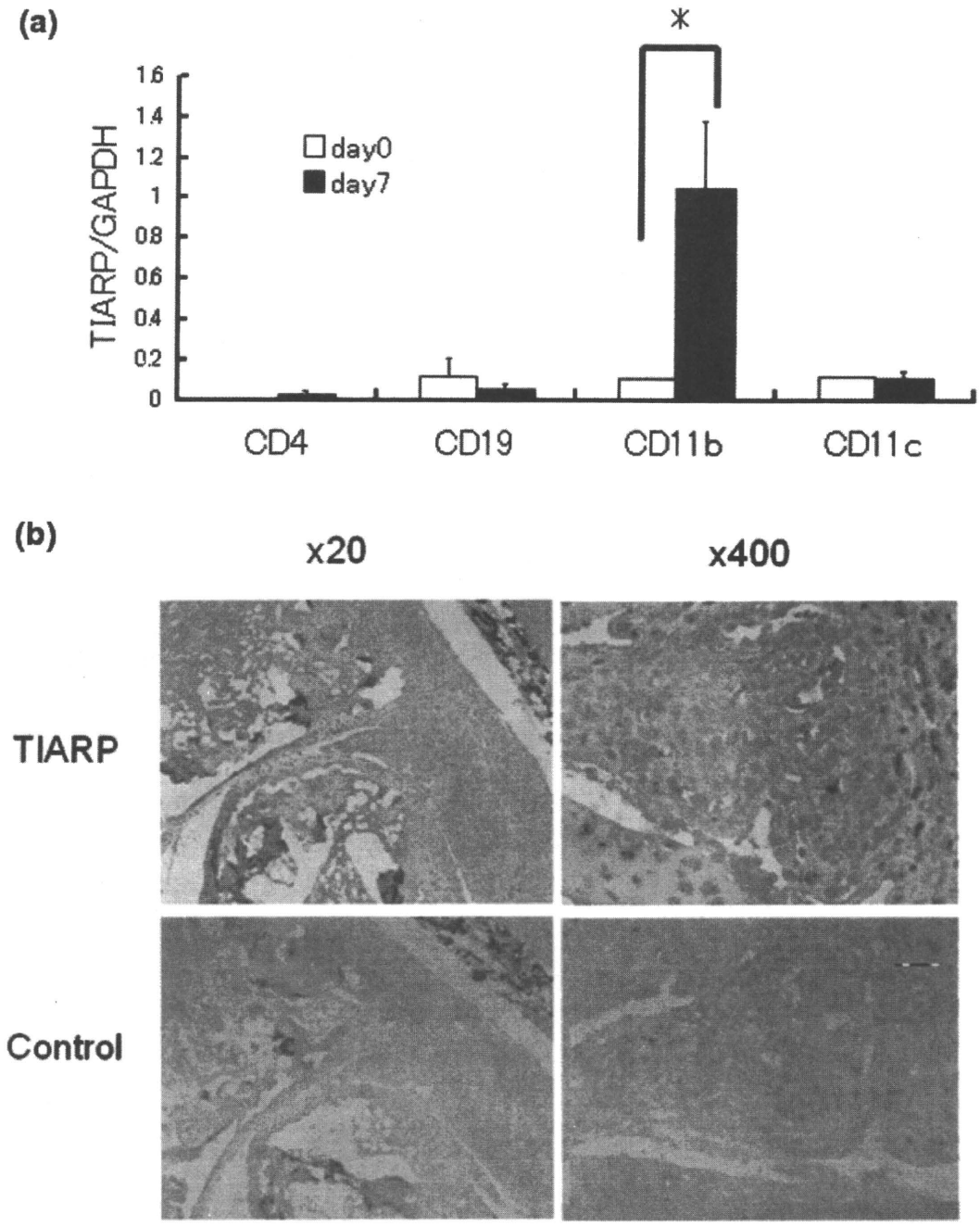
Although the therapeutic effect of TNF antagonists is confirmed in RA [1], only a few animal models of arthritis have been used to confirm the beneficial effects of TNF antagonists. For example, a recent study reported the therapeutic effect of anti-TNF mAb in DNaseII, type I interferon receptor (IFN-IR) double-knockout mice [11], although this was not a genetically unaltered mouse. Furthermore, Schubert and colleagues reported the protective effect of TNF antagonist in GPI-induced arthritis [2] and arthritis was clearly B cell-dependent [12]. We recently demonstrated the therapeutic effect of TNF antagonist in GPI-induced mice. Thus, it is important to explore TNF-regulated genes in the latter model to understand the mechanisms of action of TNFα antagonists in RA patients. When the GeneChip analysis was used, the present results showed upregulation of TIARP mRNA in the spleen of arthritic mice. TIARP was first identified as TNFα-induced cell surface protein in adipose tissues and is also known to be localized in the liver, kidney, heart, and skeletal muscle [5]. This protein was detected in the course of adipocyte differentiation and conversion and is also induced by IL-6 [6]. In this study, we confirmed its induction in $CD11b^+$ splenocytes in arthritis and we confirmed that it is upregulated in the arthritic synovium of murine GPI-induced arthritis. These findings suggest the involvement of TIARP in the process of proliferation or differentiation state induced by inflammation. In fact, previous studies indicated that TIARP is induced by TNFα and IL-6 in adipocytes [5,6]. TNFα and IL-6 are pleiotropic cytokines known to play crucial roles in human RA, and significant therapeutic effects of their antagonists have been confirmed in recent years [1,13]. In GPI-induced arthritis, both TNFα and IL-6 antagonists have protective effects [3,4], and these cytokines play important roles in the induction of arthritis in col-

Figure 3



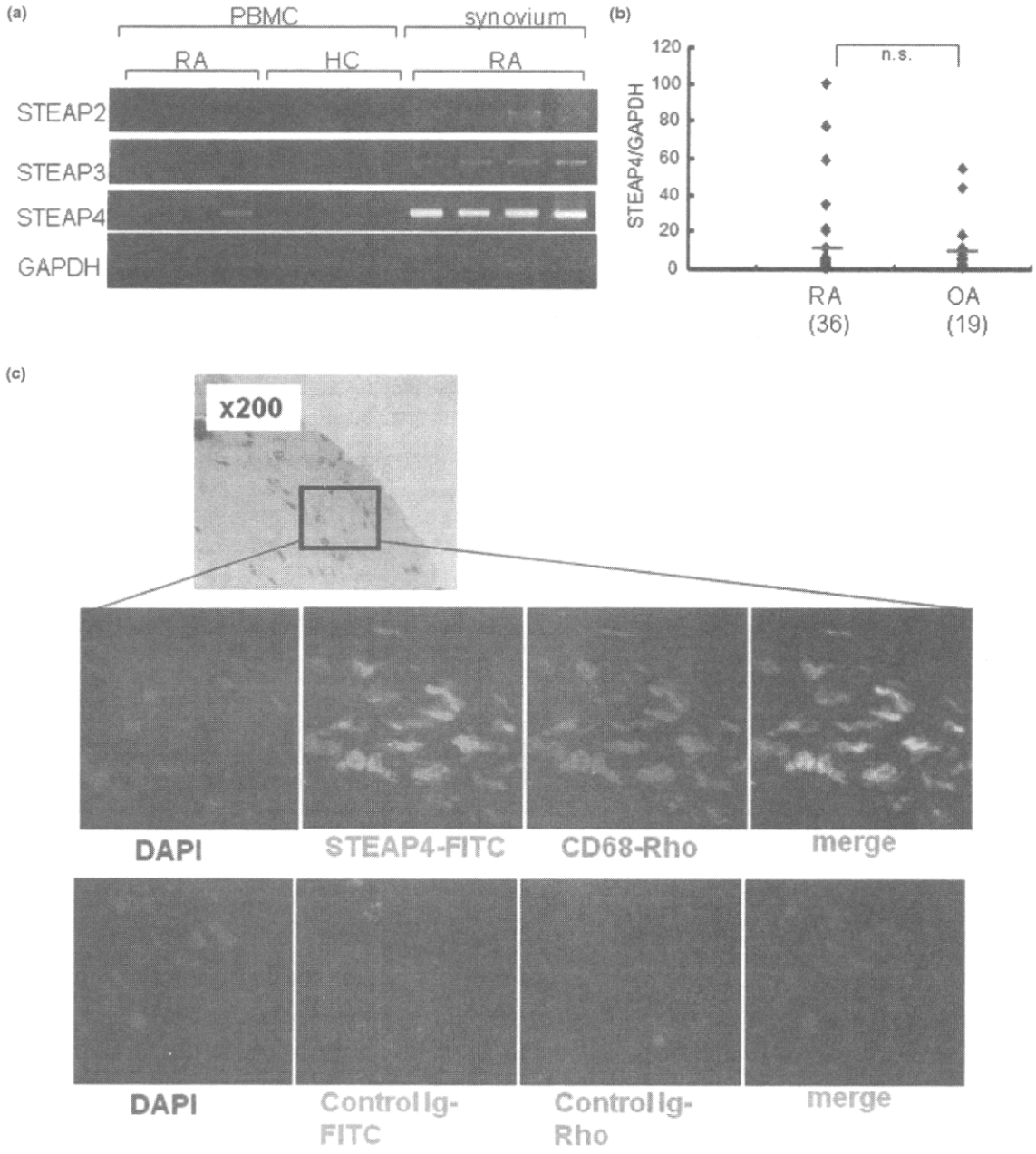
Suppression of TIARP mRNA by treatment with anti-tumor necrosis factor-alpha monoclonal antibody (anti-TNFα mAb). **(a)** The development of arthritis was blocked by administration of anti-TNFα mAb in mice immunized with glucose-6-phosphate isomerase. Data represent arthritis scores. **(b)** In spleen, administration of anti-TNFα mAb suppressed the rise in TIARP mRNA (on day 10) (solid bars), but not control Ig (open bars). However, in joints, expression of TIARP mRNA was almost comparable after the administration of anti-TNFα mAb or control Ig. Data are mean ± standard error of the mean of five mice per group. $*P < 0.05$ (Mann-Whitney *U* test). GAPDH, glyceraldehydes-3-phosphate dehydrogenase; TIARP, tumor necrosis factor alpha-induced adipose-related protein.

Figure 4



Identification of TIARP-expressing cells in splenocytes and joints of arthritic mice. **(a)** Splenocytes were isolated from naïve (day 0) mice and mice with glucose-6-phosphate isomerase (GPI)-induced arthritis and then were separated into four groups ($CD4^+$, $CD19^+$, $CD11b^+$, and $CD11c^+$) by magnetic-activated cell sorting. The expression of TIARP mRNA was analyzed by quantitative real-time polymerase chain reaction at days 0 and 7. TIARP mRNA was expressed mainly on $CD11b^+$ cells in arthritic mice. Data are mean ± standard error of the mean of five mice per group. $^*P < 0.05$ (Mann-Whitney *U* test). **(b)** Joints were obtained from mice with GPI-induced arthritis on day 14 and stained with anti-TIARP antibodies (top panels) and control antibodies (bottom panels). Inflamed synovial tissue of arthritic mice was stained with anti-TIARP antibodies. GAPDH, glyceraldehydes-3-phosphate dehydrogenase; TIARP, tumor necrosis factor alpha-induced adipose-related protein.

Figure 5



Analysis of STEAP mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) in peripheral blood mononuclear cells (PBMCs) and synovia of rheumatoid arthritis (RA) patients and healthy subjects (HC) and immunohistochemistry for STEAP4 in RA synovium. **(a)** The expression of STEAP4 mRNA and other family members (STEAP2 and STEAP3 mRNAs) was analyzed in PBMCs (RA and HC) and RA synovium using RT-PCR. In PBMCs, STEAP4 mRNA was detected in a patient with RA (1/3). Surprisingly, STEAP4 mRNA was highly expressed in all four RA synovia whereas only faint staining was noted for other members of the STEAP family. **(b)** The expression of STEAP4 mRNA in synovium with RA and osteoarthritis (OA) patients. STEAP4 mRNA expression was not statistically different between the RA and OA groups. **(c)** Co-localization of STEAP4 and CD68 in RA synovium. Images of immunohistochemistry using 4'-6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC)-anti-STEAP4, and rhodamine-anti-CD68 and a merged image are shown in the middle panels, and images with conjugated control Ig are shown in the bottom panels. Consecutive hematoxylin-and-eosin staining is shown in the top panel. GAPDH, glyceraldehydes-3-phosphate dehydrogenase; n.s., not significant; STEAP, six-transmembrane epithelial antigen of the prostate.

laboration with autoantibodies (anti-GPI antibodies) [14]. However, there is no clear scenario of balance between IL-6 and TNFα in arthritis. In TIARP knockdown animals, exposure to TNFα induced a greater amount of IL-6, suggesting a crucial role of TIARP in the balance between TNFα and IL-6 [15]. It is possible that TIARP expression plays a downregulatory role in the inflammatory cascade.

At this stage, there is no information on whether TIARP act in an antagonistic or agonistic manner with arthritis. However, one report on STAMP2 (a homolog of TIARP protein) [15] confirmed (a) upregulation of inflammatory cytokines such as TNFα and IL-6 in STAMP2-deficient mice, (b) upregulation of macrophage-specific antigens such as CD68 and CD11b, (c) infiltration of $CD68^+$ cells in adipose tissues, and (d) STAMP2-induced suppression of IL-6 expression upon stimulation by TNFα. These findings suggest that STAMP2 (TIARP) suppresses inflammatory cytokines such as TNFα and IL-6 and also blocks the activation of macrophages/monocytes.

Is this scenario applicable to patients with RA? In humans, the STEAP protein family was identified in prostate tumors [16,17] and is also known to be involved in cell apoptosis [18]. Among this family of genes, STEAP4 is highly expressed in the bone marrow, followed by placenta and fetal liver [19]. The STEAP4 expression was induced by TNFα in human adipose tissue [20] and also by TNFα in human synovial cells (our preliminary result). However, there is no report regarding the expression of this molecule in articular joints. The present study identified the expression of human ortholog STEAP4 in the synovium, especially in $CD68^+$ macrophages of patients with RA. In addition, our preliminary data using human synovial cell lines provide evidence that TNFα stimulation enhances the expression of STEAP4 protein and that a stably expressed form of STEAP4 is partially co-localized with endosomes (Tanaka and colleagues, manuscript in preparation). Further large-scale studies are required to assess the expression of STEAP4 in the joints and PBMCs of RA patients before and after treatment with TNF antagonists.

Conclusions

The results of the present study highlighted the important role of TIARP/STEAP4, a relatively new TNF-induced protein, in autoimmune arthritis in both mice and humans.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AI helped to write the manuscript, conceive of the study, perform all experiments, and coordinate statistical study. IM wrote the manuscript and conceived of the study. YT helped to perform all experiments and coordinate statistical study. KI participated in the clinical assessment. AK and NO collected the synovial samples. DG and SI participated in discussion. TS participated in the full design and coordination of the study. All authors read and approved the final manuscript.

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B cells play a crucial role as antigen-presenting cells and collaborate with inflammatory cytokines in glucose-6-phosphate isomerase-induced arthritis

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Summary

Anti-glucose-6-phosphate isomerase (GPI) antibodies from K/BxN mice directly induce arthritis; however, the transfer of these antibodies from mice with GPI-induced arthritis does not induce arthritis. CD4+ T cells play an important role in the induction and effector phase in this model; however, the roles of B cells and immunoglobulins (Igs) have not been elucidated. We investigated the roles of B cells and Igs in GPI-induced arthritis by using adoptive transfer system into SCID mice. Transfer of splenocytes of male DBA/1 mice immunized with GPI into SCID mice induced arthritis on day 6 in the latter, in association with the production of anti-GPI antibodies. Co-localization of C3 and IgG on the articular surface was identified in arthritic SCID mice. Inoculation of IgG (or anti-GPI antibodies) and CD19+-depleted splenocytes from arthritic DBA/1 mice induced arthritis in SCID mice, but not CD19+-depleted or CD4+-depleted splenocytes from DBA/1 mice. *In vitro* analysis of cytokine production by splenocytes from DBA/1 arthritic mice demonstrated production of large amounts of tumour necrosis factor (TNF)-α and interleukin (IL)-6 in an antigen-specific manner ($P < 0.01$), and production was dominated by CD19+-depleted than CD4+-depleted splenocytes ($P < 0.05$). Addition of IgG from DBA/1 arthritic mice to the culture enhanced TNF-α but not IL-6 production, and this effect was blocked by anti-Fcγ receptor antibody. *In vivo* analysis of neutralization with TNF-α protected arthritis completely in SCID mice. Our results highlight the important role of B cells in GPI-induced arthritis as autoantibody producers, and these autoantibodies can trigger joint inflammation in orchestration with inflammatory cytokines, especially TNF-α.

Keywords: animal model, autoantibodies, B cell, glucose-6-phosphate isomerase, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a common chronic autoimmune disease of unknown aetiology characterized by progressive inflammatory process and destruction of joints. Several autoantigens play a role in arthritis [1], and one of the candidate arthritogenic antigens, glucose-6-phosphate isomerase (GPI), was identified in the K/BxN model of arthritis [2]. GPI is a ubiquitous cytoplasmic enzyme, and anti-GPI antibodies in K/BxN mice induce arthritis directly. The effector mechanisms of anti-GPI antibodies have been confirmed by the requirement of innate immune system players, e.g. complement cascade, FcγR, especially FcγRIII, neutrophils and mast cells [3–6]. In addition, GPI accumulates on the synovium and joint articular surfaces, and the formation of a specific immunocomplex on the joint cavity leads ultimately to arthritis in the K/BxN serum transfer model [7]. These results indicate that ubiquitous antigens might be the targets of arthritogenic antibodies.

Recent studies have reported that immunization of DBA/1 mice with human GPI provoked arthritis, supporting the notion that autoimmunity to GPI plays a direct role in arthritis in genetically unaltered mice [8,9]. CD4+ T cells were necessary for both the induction and the effector phase of the disease because arthritis was ameliorated by depletion of CD4+ T cells with anti-CD4 monoclonal antibodies (mAbs). On the other hand, the role of B cells in this form of arthritis is still obscure. Immunoglobulin (Ig)G purified

from arthritic DBA/1 mice did not induce arthritis in naive DBA/1 mice; however, $Fc\gamma R^{-/-}$ mice developed mild arthritis following GPI immunization [8]. Moreover, both B cell-deficient C3H.Q and B10.Q mice are resistant to GPI-induced arthritis [9]. These results suggest that GPI-induced arthritis is B cell-dependent, although it is not clear that these cells are required as autoantibody-producing cells similar to antigen-presenting cells (APCs).

In the present study, we assessed the role of B cells and Igs in GPI-induced arthritis in DBA/1 mice using adoptive transfer into immunodeficient SCID mice. SCID mice were inoculated with splenocytes from GPI-immunized DBA/1 mice plus GPI. They developed arthritis with evident immune complex activation on the articular surface. Splenocytes lacking B and $CD4^+$ T cells from arthritic DBA/1 mice failed to induce arthritis in SCID mice. SCID mice recipients of both IgG (or purified anti-GPI antibodies) from GPI immunized DBA/1 mice and B cell-depleted splenocytes developed arthritis, whereas SCID mice recipients of IgG (or anti-GPI antibodies) only did not. Moreover, *in vitro* analysis of splenocytes of arthritic mice showed production of tumour necrosis factor (TNF)-α and interleukin (IL)-6 in an antigen-specific manner, driven mainly by B cell-depleted splenocytes. TNF-α, in particular, was produced mainly by $CD11b^+$ cells. *In vivo* neutralization of TNF-α protected arthritis development of SCID mice completely. These results suggest that B cells play a crucial role as antibody producers, and that antigen-induced cytokine production, especially TNF-α, seems to enhance the development of GPI-induced arthritis.

Materials and methods

Induction of GPI-induced arthritis in DBA/1 mice

Male DBA/1 mice (6–8 weeks old) were obtained from Charles River Laboratories (Yokohama, Japan). Recombinant human GPI was prepared as described previously [10]. Mice ($n = 10$) were immunized by intradermal injection of 300 μg of recombinant human GPI-gluthathione S-transfererase (GST) (hGPI) in emulsified Freund's complete adjuvant (CFA) (Difco, Detroit, MI, USA). As a control, we immunized another group of DBA/1 mice ($n = 10$) with 300 μg of GST in CFA. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Tsukuba University.

Arthritic animals were assessed clinically and ankle thickness was recorded. We used the following arthritis scoring system to evaluate the disease state (clinical score): 0 = no evidence of inflammation, 1 = subtle inflammation or localized oedema, 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, yielding a maximum possible score of 12 per mouse.

Human recombinant GPI/GST fusion protein was produced by *Escherichia coli* with pGEX vector (GE Healthcare, Uppsala, Sweden), as described previously [2]. GPI/GST fusion protein was purified from lysate with gluthathione sepharose 4B (GE Healthcare). The volume of GPI/GST fusion proteins was determined at 280 nm and the purity of proteins checked using standard sodium dodecyl sulphate gels.

Induction of arthritis in SCID mice

CB17/ICR-*Prkdc*^{scid} (SCID) mice (8–10 weeks old) were purchased from Charles River Laboratories. The spleens were removed from arthritic DBA/1 mice on day 14 after immunization. The harvested splenocytes were suspended in phosphate-buffered saline (PBS) and erythrocytes were lysed. The remaining cells were washed in PBS, then separated by magnetic affinity cell sorting (MACS; Militenyi Biotech, Bergisch Gladbach, Germany) using anti-$CD4^+$ (T cells) or anti-$CD19^+$ (B cells)-depleted splenocytes, estimated by fluorescence activated cell sorter (FACS) (> 99% cells were depleted). These cells were inoculated intraperitoneally with 100 μg GPI into SCID mice.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) microtitre plates were coated with 5 μg/ml rh-GPI in PBS (Sumitomo Bakelite, Tokyo, Japan) overnight at 4°C. The plates were then washed and saturated with 300 μl blocking solution (Dainippon Sumitomo Pharma, Tokyo, Japan) at room temperature. After 2 h, they were washed and 1/500 diluted serum with blocking solution was added. Incubation was carried out for 2 h at room temperature. The plates were washed and 150 μl alkaline phosphatase-conjugated Fc-specific anti-mouse IgG antibody (American Qualex, San Clemente, CA, USA) diluted at 1:5000 with blocking solution was added. After incubation at room temperature for 1 h, the plates were detected with 150 μl of substrate solution (9·6% 2-aminoethanol, 2·4 mM MgCl2 in distilled and deionized water, pH 9·8). Colour development was read by a microplate reader at 405 nm.

Antibody purification

Antibodies were purified from sera of DBA/1 mice immunized with 300 μg rh-GPI or GST. Serum samples were diluted 10-fold with binding buffer and then poured over a protein G column (GE Healthcare, Uppsala, Sweden) to purify IgG. Anti-GPI antibodies were also purified by affinity column (GE Healthcare), following the method described [2]. Purified antibodies were changed buffer to PBS by centricon YM-50 (Millipore, Billerica, MA, USA).

Histological examination

Mice were killed and hind-paw joints were fixed with 4% paraformaldehyde at 4°C for 6 h. The method used for

decalcification was described previously [11]. The tissues were then embedded in optimal cutting temperature compound (Miles Scientific, Naperville, IL, USA) and frozen rapidly at −80°C. Frozen sections (5-μm thick) were cut on a cryostat and placed on magnesium aluminum silicate-coated glass microscope slides and allowed to air-dry. Joints were stained with haematoxylin and eosin (H&E) or fluorescent staining. Fluorescent antibodies were anti-C3 fluorescein isothiocyanate (FITC) (ICN Biomedicals, Solon, OH, USA) and anti-IgG Texas Red (EY Laboratories, San Meteo, CA, USA).

In vitro analysis of cytokine production by splenocytes from DBA/1 arthritic mice

Spleens were removed from arthritic GPI-induced mice on day 14. The spleens were harvested and haemolyzed with 0·83% NH_4Cl, 0·12% $NaHCO_3$ and 0·004% ethylenediamine tetraacetic acid 2Na in PBS. Single-cell suspensions were prepared in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 50 μM of 2-mercaptoethanol. $CD4^+$ T cells, CD11b cells, CD11c cells or $CD19^+$ cells were isolated and enriched by MACS (Miltenyi Biotech). The cell purity was confirmed by flow cytometry (> 90%). Whole splenocytes or MACS-separated cells (1×10^6 cells/ ml) were cultured with 5 μg/ml of GPI (or GST) at 37°C in 5% CO_2 for 12 h. Anti-FcγR II/III receptor antibody (BD Bioscience, San Jose, CA, USA) was used at 1 μg/ml as an Fc blocker. Supernatants were assayed for TNF-α, interferon (IFN)-γ, IL-17 and IL-6 by Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) or ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA).

In vivo analysis using mAb for neutralizing cytokines

We used commercially available anti-TNF-α mAbs (eBioscience) and anti-IL-6 mAbs (R&D Systems) to neutralize the respective cytokines. As a control antibody, we used the same amount of Rat IgG1 isotype control (R&D Systems). In SCID-transferred arthritis, each mouse received a single injection of 100 μg of anti-TNF-α mAb, anti-IL-6 mAb or control Ig was injected on the day of splenocytes transferred (day 0).

Statistical analysis

All data were expressed as mean ± standard error of the mean. Differences between groups were examined for statistical significance using the Mann–Whitney *U*-test. A *P*-value less than 0·05 denoted a statistically significant difference.

Results

The GPI-induced arthritis in DBA/1 mice

Arthritis was induced in DBA/1 mice with 300 μg rh-GPI emulsified in CFA. Beginning on day 8 after immunization, the paws and ankles of mice were examined daily for clinical signs of arthritis. Joint swelling reached maximum around day 14, then resolved gradually (Fig. 1a). Arthritic changes were observed mainly in the paws (Fig. 1b, right) and ankles of immunized DBA/1 mice, but not in digits. Control (GST) immunization did not lead to apparent arthritis (Fig. 1b,c, left). Histopathological examination showed synovium proliferation (Fig. 1c, right), resulting in bone destruction (data not shown). Immunohistochemical analysis confirmed co-localization of IgG and C3 on the surface of cartilage on day 14 in arthritic DBA/1 mice (Fig. 1d right; control immunization on left). These findings suggest that immune complex activation in local joints is involved in the development of GPI-induced arthritis.

Successful transfer of GPI-induced arthritis into immunodeficient mice

Splenocytes (1×10^7 cells) from arthritic DBA/1 mice were inoculated into SCID mice on day 14 post-immunization with 100 μg of GPI. Spleens from control SCID mice (Fig. 2a left) or SCID mice inoculated with splenocytes (1×10^7 cells) from arthritic DBA/1 mice (Fig. 2a right, on day 14) was shown. Arthritis developed in splenocyte-inoculated SCID mice (Fig. 2b right, c). However, arthritis was not observed in both SCID mice inoculated with the same number of splenocytes from arthritic DBA/1 mice without GPI, and SCID mice inoculated with splenocytes from naive DBA/1 mice with GPI (Fig. 2b left, c) These results indicate that splenocytes from arthritic mice plus GPI contain important factor(s) in the induction of arthritis.

Histological analysis of arthritic SCID mice

Histopathological examination of the arthritic joints of SCID mice showed synovial hyperplasia in arthritic SCID mice inoculated with splenocytes from arthritic DBA/1 mice (H&E staining, Fig. 3b,c), but not in SCID mice inoculated with splenocytes from naive DBA/1 mice (Fig. 3a). Immunohistochemical study showed co-localization of IgG and C3 on the cartilage surface of arthritic SCID mice (Fig. 3d), but not in joints of SCID mice inoculated with splenocytes from naive DBA/1 mice. These findings suggest that Igs produced by inoculated splenocytes from DBA/1 mice attach to the articular surface of SCID mice and result in arthritis by complement activation.

Importance of T and B cells in arthritis of SCID mice

To evaluate the role of $CD19^+$ or $CD4^+$ cells in arthritis of SCID mice, we inoculated 1×10^7 $CD19^+$- or $CD4^+$-depleted splenocytes of arthritic DBA/1 mice plus 100 μg of GPI into SCID mice. In these inoculi, the percentage of $CD19^+$ and $CD4^+$ cells in depleted splenocytes was less than 1%. Neither

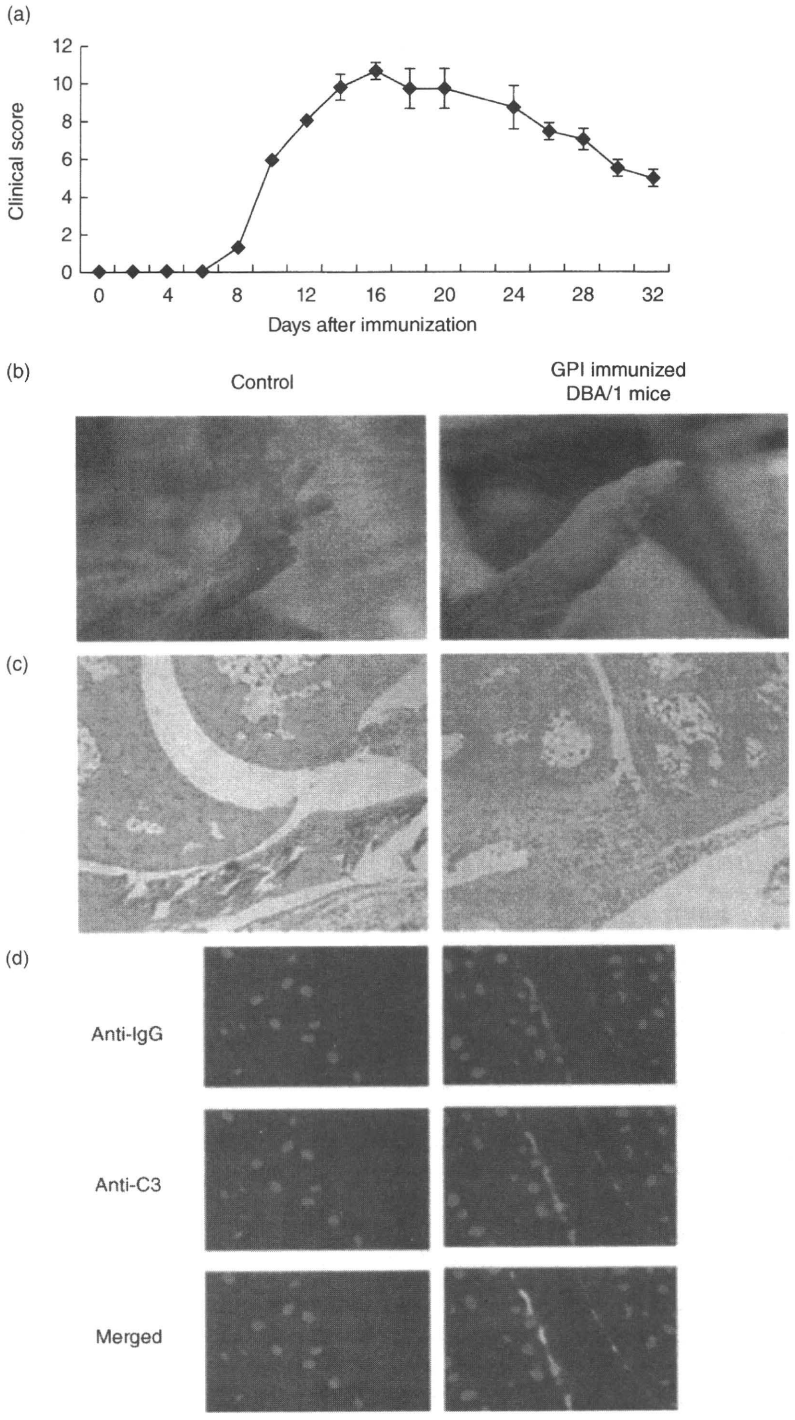

Fig. 1. Clinical and histological evaluation of glucose-6-phosphate isomerase (GPI)-induced arthritis in DBA/1 mice. Mean of clinical score (a) (±standard error of the mean, 10 mice) followed days after immunization. (b) Paw of control DBA/1 mouse treated with control antigens [gluthathione S-transfererase (GST)] 300 μg (left). DBA/1 mice were immunized with rh-GPI 300 μg in Freund's complete adjuvant (CFA) (right). (c) Histological examination of ankle joints of the control (left) and GPI-induced arthritis on day 14 showing severe synovium proliferation (haematoxylin and eosin staining, right). (d) Anti-C3 (green) and anti-immunoglobulin (Ig)G (red) staining in joints of control (left) and arthritic DBA/1 mice (right). Nuclei were counterstained with 4,6-diamino-2-phenylindole (blue). C3 and IgG were co-localized on the surface of cartilage of ankle joints (right). Magnification of original photographs: ×40 (c) or ×600 (d); spl: splenocytes.

$CD19^+$ nor $CD4^+$ cell-depleted splenocytes induced arthritis in SCID mice (Fig. 4a), or produced anti-GPI antibodies (Fig. 4b), suggesting that both $CD19^+$ and $CD4^+$ cells play important roles in the induction of arthritis in SCID, and that production of anti-GPI antibodies may be indispensable for such induction.

Importance of B cells as producers of antibodies in arthritis of SCID mice

It has been reported previously that B cell-deficient mice are resistant to GPI-induced arthritis [9]. However, whether these cells act as autoantibody-producing cells as well as APCs

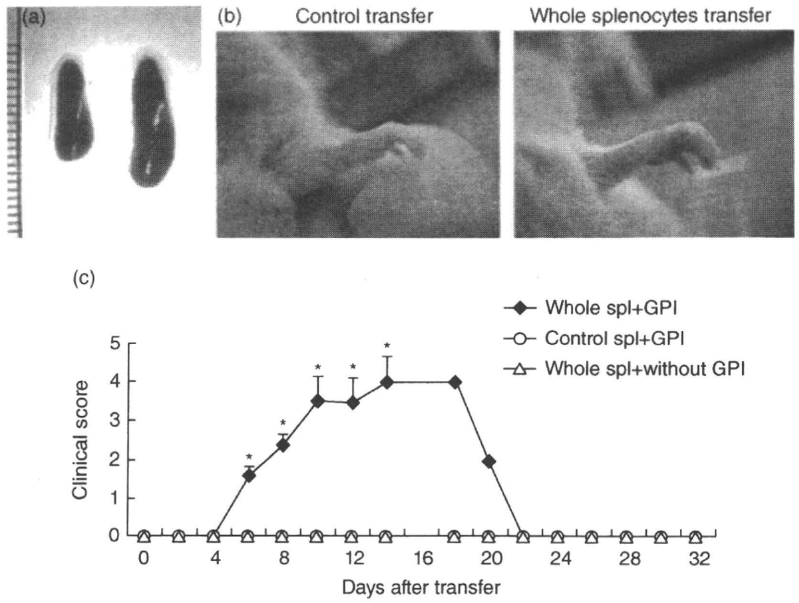

Fig. 2. Transfer of arthritis in SCID mice. Glucose-6-phosphate isomerase (GPI)-immunized DBA/1 mice were killed on day 14, and 1×10^7 splenocytes (spl) were isolated and transferred into SCID mice with 100 μg of GPI. (a) Spleen of control SCID mice (left) and SCID mice inoculated with splenocytes of arthritic DBA/1 mice 1×10^7 cells (right on day 14). (b) Feet of SCID mice inoculated with splenocytes from naive DBA/1 mice (left) and SCID mice inoculated with splenocytes from GPI-induced arthritic DBA/1 mice (right). (c) Clinical score and development of arthritis in SCID mice. Swelling of paws was observed on day 6 in SCID mice inoculated with whole splenocytes plus 100 μg of GPI (◆). Control mice were inoculated with 1×10^7 splenocytes only from immunized DBA/1 mice (△) or 1×10^7 splenocytes from control immunized DBA/1 mice plus 100 μg of GPI (○). Control mice did not develop arthritis. Data are mean ± standard error of the mean of five mice in each group. $^*P < 0.05$ by Mann–Whitney *U*-test.

is unknown at present. To investigate the role of autoantibodies, we inoculated SCID mice with IgGs from arthritic DBA/1 mice. IgGs were purified from the sera of DBA/1 mice on day 14 after immunization. Injection of 3 mg IgG alone from arthriticDBA/1 mice did not result in overt arthritis in SCID mice, even if we added 100 μg of GPI (Fig. 5a). However, injection of IgG with $CD19^+$-depleted splenocytes and GPI resulted in the development of arthritis in SCID mice (Fig. 5a). To investigate further the arthritogenicity of anti-GPI antibodies, we used affinity purified anti-GPI antibodies from arthritic DBA/1 mice. Injection of 3 mg anti-GPI antibodies alone did not result in arthritis in SCID mice, even if we added 100 μg of GPI (Fig. 5b). However, with $CD19^+$-depleted splenocytes, even if we used 1mg of affinity purified anti-GPI antibodies from GPI-induced mice instead of IgG, clear arthritis was developed in SCID mice (Fig. 5b). These findings suggest that $CD19^+$ cells play an important role as producers of antibody (especially anti-GPI antibodies) in arthritis of SCID mice; however, anti-GPI antibodies alone from GPI-induced arthritis do not have arthritogenecity.

Importance of TNF-α in the development of arthritis in SCID mice

To determine the humoral factors that were mediated by arthritis with splenocytes from GPI-induced arthritis plus GPI in SCID mice, we screened *in vitro* cytokine production from splenocytes plus GPI. We selected two proinflammatory cytokines in these experiments based on the preliminary results of cytometric beads array analysis, which revealed antigen-specific expression of TNF-α and IL-6 (data not shown); they have recently proved to be important in the induction of GPI-induced arthritis [12]. Indeed, the addition of GPI to the culture medium induced the production of large amounts of TNF-α and IL-6, while control antigen did not induce these cytokines (Fig. 6a). We also examined the production of these cytokines by $CD19^+$- and $CD4^+$-depleted cells. TNF-α and IL-6 levels were enhanced in the presence of $CD19^+$-depleted cells compared with $CD4^+$-depleted cells (Fig. 6a), and enriched slightly in $CD19^+$-depleted cells compared with whole splenocytes. To examine the role of IgG from DBA/1 arthritic mice, $CD19^+$-depleted splenocytes were stimulated with GPI and/or IgG *in vitro*. IgG triggered weak production of TNF-α and Fcγ blockade suppressed TNF-α production (Fig. 6b, $P < 0.05$). On the other hand, IL-6 production was regulated by neither IgG nor Fcγ blockade (Fig. 6b). To confirm the dependency of these inflammatory cytokines of arthritis in SCID mice, neutralizing mAbs were injected *in vivo* on the day of inoculation of splenocytes. Surprisingly, anti-TNF-α mAb protected arthritis completely in SCID mice, whereas anti-IL-6 mAb blocked arthritis partially (Fig. 6c). These findings suggest

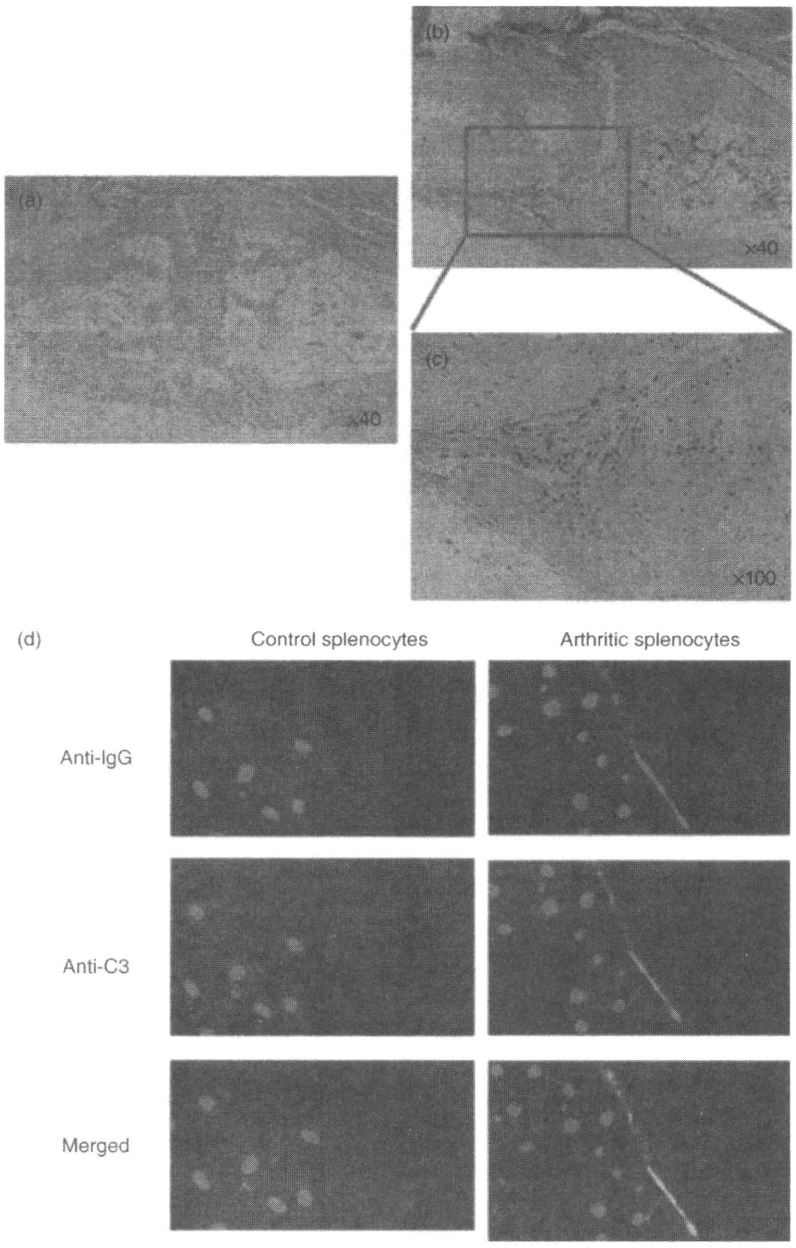

Fig. 3. Histological evaluation of joints of SCID mice. Joints of control mice inoculated splenocyte (spl) from naive DBA/1 mice with 100 μg of glucose-6-phosphate isomerase (GPI). (b,c) Synovial hyperplasia in a representative SCID mouse inoculated with splenocytes from arthritic DBA/1 mice. (d, right) Co-localization of immunoglobulin (Ig)G (red) and C3 (green) on the articular surface of SCID mice on day 14 after transfer by fluorescent staining. Nuclei were counterstained with 4,6-diamino-2-phenylindole (blue). Magnification of original photographs: ×40 (a), ×100 (b, c) or ×600 (d).

that TNF-α in particular (partially IL-6) induced by GPI may contribute to the development of arthritis, although IgG from arthritic mice contributed weakly to the production of TNF-α via Fcγ receptors.

CD11b$^+$ cells collaborating with CD4$^+$ T cells produce predominantly TNF-α

To analyse further the dominant cell populations that can produce TNF-α and IL-6, MACS-separated cells were co-cultured with GPI or GST (Fig. 7a,b). TNF-α was produced by several cell populations, driven mainly by CD11b$^+$ cells (Fig. 7a). It is possible that TNF-α production from CD11b cells was induced by the collaboration of activated T cells containing CD11b when cultured with GPI. On the other hand, IL-6 was produced predominantly by CD11c$^+$ cells (Fig. 7b). This cytokine production was enhanced by adding CD4$^+$ cells ($P < 0{\cdot}05$), thus CD4$^+$ T cells might also contribute for producing inflammatory cytokines.

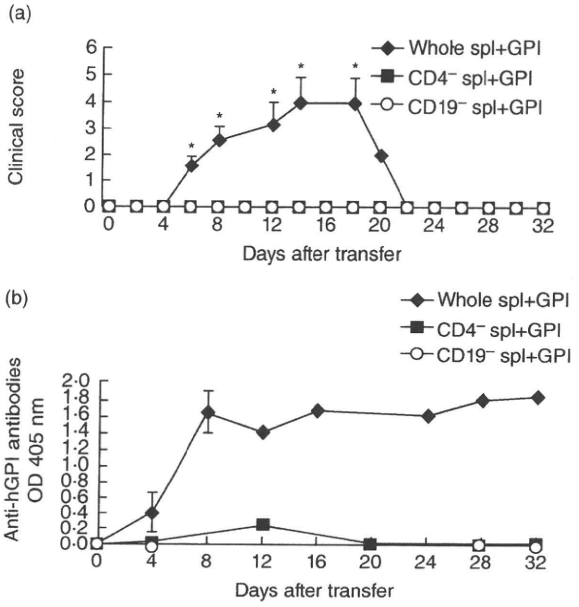

Fig. 4. Importance of anti-GPI antibodies in transfer of arthritis. CD19-depleted or $CD4^+$-depleted splenocytes (spl) from arthritic DBA/1 mice obtained on day 14 after immunization were inoculated with glucose-6-phosphate isomerase (GPI) into SCID mice. (a) Mean clinical score. (b) Anti-GPI antibodies detected by enzyme-linked immunosorbent assay (ELISA) at 405 nm (b). (◆) SCID mice that received 1×10^7 of splenocytes from arthritic DBA/1 mice plus 100 μg GPI; (■) SCID mice recipients of 1×10^7 $CD4^+$-depleted cells plus 100 μg GPI; (○) SCID mice recipients of 10^7 CD19-depleted cells plus 100 μg GPI. Data are mean ± standard error of the mean of five mice in each group. $^*P < 0{\cdot}05$ by Mann–Whitney *U*-test.

Exploring antigen-presenting function of B cells

Finally, to evaluate B cell function as APCs, MACS-separated $CD4^+$ T cell and $CD19^+$ cells were co-cultured with GPI. IFN-γ and IL-17 production were used to indicate the barometer of the antigen presentation function of B cells. Both IFN-γ and IL-17 were up-regulated clearly by adding $CD19^+$ splenocytes ($P < 0{\cdot}05$), indicating that $CD19^+$ cell may function as APCs (Fig. 7c,d).

Discussion

Anti-GPI antibodies from K/BxN mice are well known as arthritogenic autoantibodies, and their effector mechanisms have been identified in several elegant studies [2–7]. Briefly, the key players involved in the development of arthritis after the transfer of anti-GPI antibodies included Fcγ receptor (particularly FcγRIII), alternative complement pathways such as factors B, C3, C5 and C5aR [3], subsets of Fcγ receptor or C5a receptor-bearing cells [4–6] and some inflammatory cytokines such as IL-1 and TNF-α [3]. In particular, a dominant pathological action driven by anti-GPI antibodies is a local association between GPI and anti-GPI on the articular surface, which leads to complement activation in the joints [7,13].

However, anti-GPI antibodies from GPI-induced arthritis did not induce overt arthritis in naive mice [8]. A previous report showed that B cell-deficient C3H.Q and B 10.Q mice were resistant to GPI-induced arthritis [9]. Moreover, FcγR-deficient mice were protected from GPI-induced arthritis, whereas mice deficient in inhibitory FcγRIIB developed severe arthritis [8]. These results show that B cells play an essential role in arthritis by producing autoantibodies that

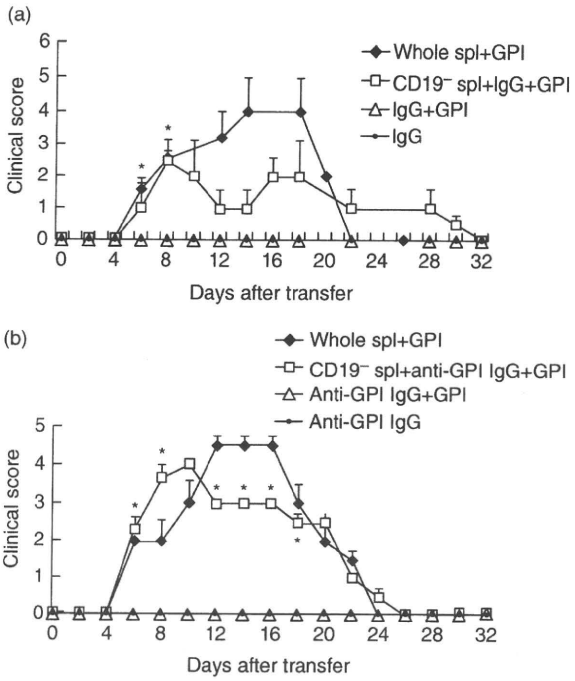

Fig. 5. Role of B cells in induction of arthritis in SCID mice. IgG from arthritic DBA/1 mice [alone or with glucose-6-phosphate isomerase (GPI)] or immunoglobulin (Ig)G plus $CD19^+$-depleted cells were inoculated into SCID mice. Development of arthritis in SCID mice was monitored. (a) Mean clinical score is depicted. (◆) SCID mice that received 1×10^7 splenocytes (spl) plus 100 μg GPI; (□) SCID mice recipients of 1×10^7 CD19-depleted cells from arthritic DBA/1 mice with 3 mg of IgG plus 100 μg of GPI; (△) SCID mice recipients of 3 mg of IgG from arthritic DBA/1 mice plus 100 μg GPI; (•) SCID mice recipients of 3 mg of IgG from arthritic DBA/1 mice alone. (b) Affinity purified anti-GPI antibodies from arthritic DBA/1 mice (alone or with GPI), or anti-GPI antibodies plus $CD19^+$-depleted cells were inoculated into SCID mice and monitored. (◆) SCID mice that received 1×10^7 spenocytes plus 100 μg GPI; (□) SCID mice recipients of 1×10^7 CD19-depleted cells from arthritic DBA/1 mice with 1 mg of anti-GPI antibodies plus 100 μg of GPI; (△) SCID mice recipients of 3 mg of anti-GPI antibodies from arthritic DBA/1 mice plus 100 μg GPI; (•) SCID mice recipients of 3 mg of anti-GPI antibodies from arthritic DBA/1 mice alone. Data are mean ± standard error of the mean of five mice in each group. $^*P < 0{\cdot}05$ by Mann–Whitney *U*-test.

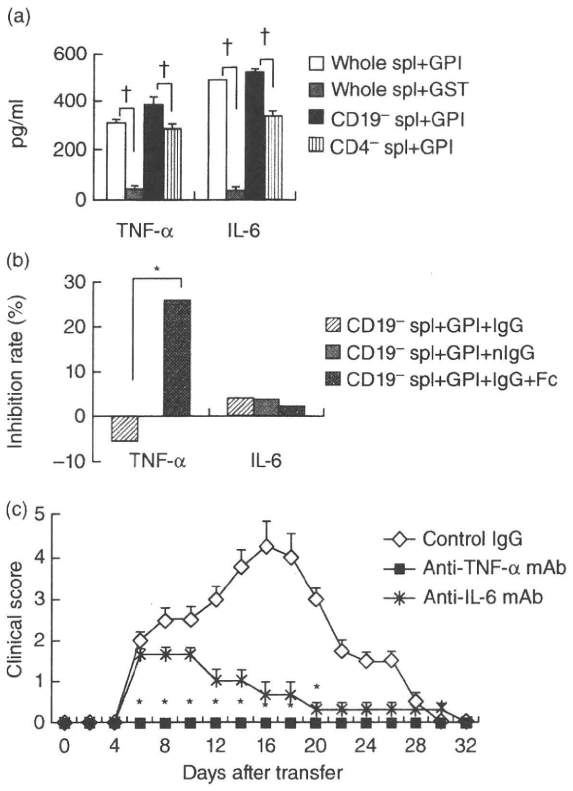

Fig. 6. *In vitro* cytokine production by splenocytes from arthritic DBA/1 mice and *in vivo* neutralization of inflammatory cytokines in SCID mice. Cytokine concentrations in supernatant of cultured splenocytes (spl) from arthritic DBA/1 mice were assessed by enzyme-linked immunosorbent assay. (a) Whole splenocytes or separated splenocytes (10^6 cell/ml) were cultured with 5 μg/ml of glucose-6-phosphate isomerase (GPI) or gluthathione S-transfererase (GST). (b) $CD19^+$-depleted splenocytes were cultured with GPI and immunoglobulin (Ig)G with/without FcγR blocker. IgG was purified from arthritic DBA/1 mice on day 14 after GPI immunization. Naive IgG (nIgG) was purified from naive DBA/1 mice. Inhibition rate was calculated to be divided by amount of productions from $CD19^+$-depleted splenocytes stimulated with GPI. Representative data of three independent experiments with three individual mice per experiment. (c) Neutralization of inflammatory cytokines was performed *in vivo* by monoclonal antibody (mAb), five mice in each group. SCID mice recipients of 1×10^7 splenocytes from arthritic DBA/1 mice plus 100 μg GPI and 100 μg of control IgG (◇), anti-tumour necrosis factor (TNF)-α mAb (■) or anti-interleukin (IL)-6 mAb (*). Data are mean ± standard error of the mean of five mice in each group. $^*P < 0{\cdot}05$, $^\dagger P < 0{\cdot}01$, by Mann–Whitney *U*-test.

result in FcγR activation in this model. In our immunohistological study, a clear complement activation by immune complex was observed in joints of mice with GPI-induced arthritis. This finding suggests that local immune complex (probably GPI–anti-GPI antibodies) activation in the joints also plays an important role in GPI-induced arthritis.

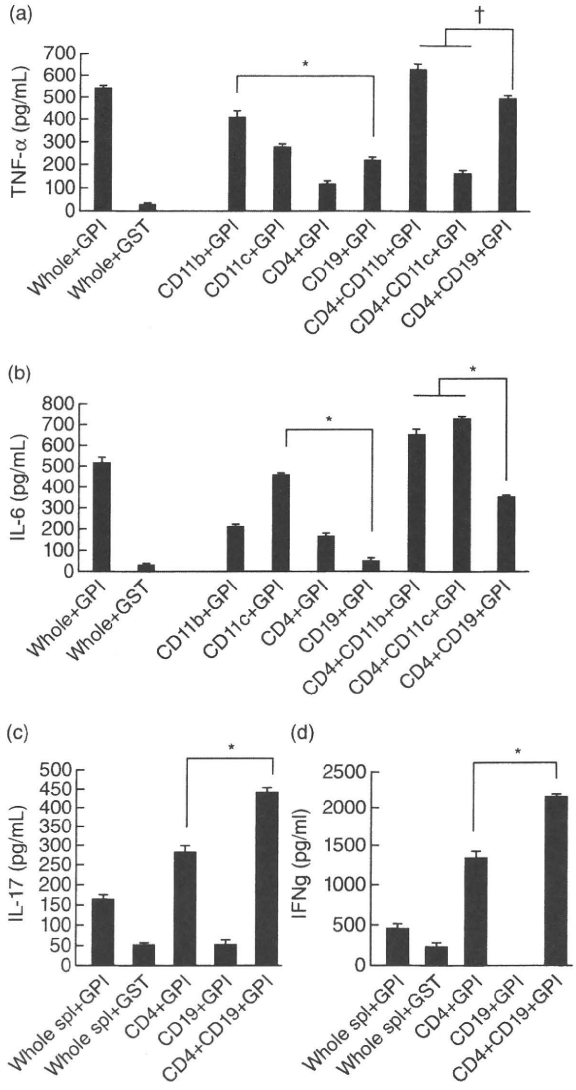

Fig. 7. Exploration of dominant cell population of inflammatory cytokines, and B cell functions as an antigen-presenting cells (APCs) *in vitro*. Whole splenocytes (spl) or independent magnetic affinity cell sorting (MACS) separated ($CD4^+$, $CD19^+$, $CD11b^+$ and $CD11c^+$ cells) splenocytes (total 1×10^6 cells/ml) were cultured with 5 μg/ml of glucose-6-phosphate isomerase (GPI) or gluthathione S-transfererase (GST). Inflammatory cytokines [tumour necrosis factor (TNF)-α (a) and interleukin (IL)-6 (b)] and T cell-secreted cytokines [IL-17 (c) and interferon (IFN)-γ (d)] were compared between $CD19^+$ cells and other APCs ($CD11b^+$ $CD11c^+$ cells). Cytokine concentrations in supernatant of cultured splenocytes from arthritic DBA/1 mice were assessed by enzyme-linked immunosorbent assay. The purity of cells was estimated by fluorescence activated cell sorter flow cytometry (> 90%). Data are mean ± standard error of the mean of three mice in each group. $^*P < 0{\cdot}05$, $^\dagger P < 0{\cdot}001$, by Mann–Whitney *U*-test.

To determine the role of B cells more precisely, we set up a transfer system using SCID mice. SCID mice inoculated with splenocytes from GPI-immunized DBA/1 mice together with GPI developed arthritis, and the immune complex activation was also noted on the articular surface of SCID mice. In GPI-induced arthritis, the expression of TNF-α mRNA in inflammatory joints and serum was increased on day 7 when detectable amounts of anti-GPI antibodies were produced (reference [12] and our unpublished data). B cell-depleted splenocytes from arthritic DBA/1 mice could not induce arthritis in SCID mice. On the other hand, SCID mice that received IgG (or anti-GPI antibodies) with B cell-depleted splenocytes from GPI-immunized DBA/1 mice developed arthritis, whereas SCID mice that received only IgG did not. These results suggest that B cells play a crucial role as antibody producers, followed by immune complex deposition on the articular surfaces in arthritis.

Our scenario is similar to adoptive transfer of collagen-induced arthritis (CIA) to SCID mice [14–16]. However, the GPI-induced arthritis in SCID mice occurred earlier (5–6 days) than CIA (14–16 days). The other difference between these two types of arthritis is that IgG from GPI-induced arthritis did not have arthritogenic capacity, whereas CIA IgG alone exhibit such capacity. Thus, anti-GPI antibodies produced by inoculated splenocytes play an important role in the induction of arthritis. However, these antibodies could not induce arthritis when injected alone, and thus we need to know about other humoral factors that trigger arthritis.

Our *in vitro* assay with splenocytes from GPI-induced arthritis plus GPI indicated that TNF-α and IL-6 may be crucial for the induction of arthritis. An earlier study from our laboratories identified the therapeutic efficacy of both anti-TNF-α mAb and anti-IL-6 mAb in GPI-induced arthritis [12]. Moreover, we clearly confirmed a protective effect of anti-TNF-α mAb in SCID-transferred arthritis. These results indicate that arthritis in SCID recipients may be enhanced mainly not only by anti-GPI antibodies, but also humoral factors such as TNF-α and IL-6. In particular, the development of arthritis was associated with the production of anti-GPI antibodies in SCID mice, thus autoantibodies might play a crucial role especially in the local joints, collaborating with inflammatory cytokines.

Concerning the other role of B cells, our *in vitro* assay suggests that B cells had a weak capacity of producing TNF-α, as well as antigen-presenting function with GPI culture. A recent paper reported that subsets of dendritic cells (DC) could express CD19 [17], thus it is possible that these cells comprise such functions of B cells. However, we tested *in vivo* experimentally with $CD19^+$-depleted cells, suggesting that the antoantibody produced indeed contributed to the development of arthritis.

What is the role of T cells in GPI-induced arthritis? Based on our experiments, splenocytes lacking $CD4^+$ cells failed to induce arthritis in SCID mice. The lack of anti-GPI antibodies in the serum of SCID recipients of the $CD4^+$ T cell-depleted cell population suggests that production of autoantibodies is $CD4^+$ T cell-dependent. Moreover, our *in vitro* assay identified $CD19^+$-depleted cells (probably comprising APCs plus T cells) as the main source of inflammatory cytokines that can trigger arthritis. TNF-α and IL-6 production was enhanced by adding $CD4^+$ cells, as confirmed by *in vitro* assay. In this regard, in GPI-induced arthritis, administration of anti-CD4 mAb on days 11 and 14 after immunization induced rapid remission of the arthritis [8]. We have reported previously that GPI-specific $CD4^+$ T cells were differentiated to T helper type 1 (Th1) and Th17 [18]. The administration of anti-IL-17 mAb on day 7 ameliorated arthritis significantly, whereas that administered on day 14 did not affect the disease. Moreover, *our vitro* assay using splenocytes on day 14 could detect tiny amounts of IL-17 with GPI ([17], and our unpublished data). These findings show that $CD4^+$ T cells (particularly Th17 cells) are necessary in the induction phase, and they function as supporters of production with autoantibodies and inflammatory cytokines in the effector phase of GPI-induced arthritis.

Are these scenarios relevant to human RA? High titres of anti-GPI antibodies are found in patients with severe forms of RA, but in only a few control individuals [10,19,20]. We reported recently that a *FCGR3A*-158V/F functional polymorphism was associated with RA in anti-GPI antibody-positive individuals, because 89% of healthy subjects positive for anti-GPI antibodies possessed homozygous low-affinity genotype *FCGR3A*-158F [21]. Moreover, among anti-GPI antibody-positive individuals, GPI-reactive $CD4^+$ T cells, especially Th1 cells, are detected specifically in peripheral blood mononucleocytes of patients with RA who share either human leucocyte antigen (HLA)-DRB1 *0405 or *0901 haplotypes [22]. These findings suggest that arthritis in anti-GPI antibody-positive individuals depends on several important factors, such as GPI-reactive T cells, HLA-DR*0405/*0901 and FcγRIII.

What of the role of anti-GPI antibodies in GPI-induced arthritis? The $H2^q$ haplotype confers severe form of arthritis [9]. High titres of anti-GPI antibodies were also found in arthritis-resistant C57BL/6($H2^b$) mice, although their T cells had weak GPI responses ([8], and our observations) compared with arthritis-susceptible DBA/1 mice. In addition, $Fc\gamma R^{-/-}$ mice are protected from GPI-induced arthritis, whereas $Fc\gamma RIIB^{-/-}$ mice developed pronounced arthritis [8]. These findings indicate that anti-GPI antibodies do not induce arthritis *per se*; it is probable that unique activation of major histocompatibility complex class II and antigen-specific T cells might be indispensable. In this regard, GPI-induced arthritis appears to be akin to human RA.

In conclusion, we identified that B cells play a crucial role in GPI-induced arthritis as autoantibody producers. This finding might explain how autoantibodies orchestrate the induction of arthritis with inflammatory cytokines such as TNF-α in patients with RA.

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Research article

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Arthritogenic T cell epitope in glucose-6-phosphate isomerase-induced arthritis

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Abstract

Introduction Arthritis induced by immunisation with glucose-6-phosphate isomerase (GPI) in DBA/1 mice was proven to be T helper (Th) 17 dependent. We undertook this study to identify GPI-specific T cell epitopes in DBA/1 mice (H-2q) and investigate the mechanisms of arthritis generation.

Methods For epitope mapping, the binding motif of the major histocompatibility complex (MHC) class II (I-Aq) from DBA/1 mice was identified from the amino acid sequence of T cell epitopes and candidate peptides of T cell epitopes in GPI-induced arthritis were synthesised. Human GPI-primed CD4+ T cells and antigen-presenting cells (APCs) were co-cultured with each synthetic peptide and the cytokine production was measured by ELISA to identify the major epitopes. Synthetic peptides were immunised in DBA/1 mice to investigate whether arthritis could be induced by peptides. After immunisation with the major epitope, anti-interleukin (IL) 17 monoclonal antibody (mAb) was injected to monitor arthritis score. To investigate the mechanisms of arthritis induced by a major epitope, cross-reactivity to mouse GPI peptide was analysed by flow cytometry and anti-GPI antibodies were measured by ELISA. Deposition of anti-GPI antibodies on the cartilage surface was detected by immunohistology.

Results We selected 32 types of peptides as core sequences from the human GPI 558 amino acid sequence, which binds the binding motif, and synthesised 25 kinds of 20-mer peptides for screening, each containing the core sequence at its centre. By epitope mapping, human GPI325–339 was found to induce interferon (IFN) γ and IL-17 production most prominently. Immunisation with human GPI325–339 could induce polyarthritis similar to arthritis induced by human GPI protein, and administration of anti-IL-17 mAb significantly ameliorated arthritis ($p < 0.01$). Th17 cells primed with human GPI325–339 cross-reacted with mouse GPI325–339, and led B cells to produce anti-mouse GPI antibodies, which were deposited on cartilage surface.

Conclusions Human GPI325–339 was identified as a major epitope in GPI-induced arthritis, and proved to have the potential to induce polyarthritis. Understanding the pathological mechanism of arthritis induced by an immune reaction to a single short peptide could help elucidate the pathogenic mechanisms of autoimmune arthritis.

Introduction

Rheumatoid arthritis (RA) is characterised by symmetrical polyarthritis and joint destruction. Although the aetiology is considered to be autoimmune reactivity to some antigens, the exact mechanisms are not fully understood. So far, several models of arthritis have been described and analysed to understand the aetiological mechanisms of RA. Glucose-6-phosphate isomerase (GPI)-induced arthritis, a murine model of RA, is induced by immunisation with recombinant human (rh) GPI of DBA/1 mice [1]. We have previously demonstrated

APC: antigen-presenting cell; CIA: collagen-induced arthritis; CII: type II collagen; CTLA-4 Ig: cytotoxic T-lymphocyte antigen 4 immunoglobulin; DAPI: 4',6-diamidino-2-phenyindole, dilactate; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; GPI: glucose-6-phosphate isomerase; IFN: interferon; IL: interleukin; mAb: monoclonal antibody; MHC: major histocompatibility complex; PBS: phosphate-buffered saline; RA: rheumatoid arthritis; rh: recombinant human; SD: standard deviation; SEM: standard error of the mean; TCR: T cell receptor; Th: T helper.

that the T helper (Th) 17 subset of CD4+ T cells play a central role in the pathogenesis of GPI-induced arthritis; GPI-specific CD4+ T cells were skewed to Th17 at the time of onset, and blockade of interleukin (IL) 17 resulted in a significant amelioration of arthritis [2]. Furthermore, the data that the administration of cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4 Ig) in the effector phase ameliorated the progress of arthritis implies the importance of Th17 cells even in the effector phase [3].

In this study, we further explored the epitopes of GPI-specific CD4+ T cells and identified human GPI $(hGPI)_{325-339}$ as a major epitope. Interestingly, the amino acid sequence of $hGPI_{325-339}$ (IWYINCFGCETHAML) was the same as that of bovine (type II collagen) $CII_{256-270}$(GEPGIAGFKGEQGPK), the dominant epitope of collagen-induced arthritis (CIA), at the major histocompatibility complex (MHC) binding sites [4]. Of note is that arthritis similar to GPI-induced arthritis was generated by immunisation with a short 15-mer single peptide in genetically unaltered mice. By analysis of peptide-induced arthritis, we found that $hGPI_{325-339}$-primed Th17 cells reacted with mouse GPI $(mGPI)_{325-339}$ peptide and subsequently lead to the production of anti-mouse GPI antibodies, which deposited over the cartilage surface of inflaming joints. Our findings should be helpful in unravelling the mechanism of autoimmune arthritis.

Materials and methods

Mice

DBA/1 mice were purchased from Charles River Laboratories, Japan. All mice were kept under specific pathogen-free conditions and all experiments were conducted in accordance with the University of Tsukuba ethical guidelines.

GPI and synthetic peptides

Recombinant mouse GPI and rhGPI were prepared as described previously [5,6]. Briefly, human GPI or mouse GPI cDNA was inserted into the plasmid pGEX-4T3 (Pharmacia, Uppsala, Sweden) for expression of glutathione S-transferase-tagged proteins. *Escherichia coli* harboring the pGEX-hGPI plasmid was allowed to proliferate at 37°C, before 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium, followed by further culture overnight at 30°C. The bacteria were lysed with a sonicator and the supernatant was purified with a glutathione-sepharose column (Pharmacia, Uppsala, Sweden). The purity was estimated by SDS-PAGE.

Crude peptides were synthesised for epitope screening by Mimotopes (Melbourne, Victoria, Australia), and peptides with 90% purity were synthesised for a major epitope decision and induction of arthritis by Invitrogen (Carlsbad, CA). Candidate peptides, which were thought to bind the binding motif, were selected with web soft MHCPred (The Jenner Institute, Oxford, UK) [7].

Induction of arthritis

DBA/1 mice were immunised with 300 μg rhGPI for GPI-induced arthritis, or 10 μg or 25 μg synthetic peptide for peptide-induced arthritis in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The rhGPI and synthetic peptide were emulsified with complete Freund's adjuvant at a 1:1 ratio (v/v). For induction of arthritis, 150 μl of the emulsion was injected intradermally at the base of the tail of the mouse. On days 0 and 2 after immunisation, 200 ng of pertussis toxin was injected intraperitoneally to develop peptide-induced arthritis. The arthritis score was evaluated visually using a score of 0 to 3 for each paw. A score of 0 represented no evidence of inflammation, 1 represented subtle inflammation or localised oedema, 2 represented easily identified swelling but localised to either the dorsal or ventral surface of the paws, and 3 represented swelling in all areas of the paws.

Treatments of arthritis with anti-IL-17 monoclonal antibodies

To neutralise IL-17, mice were injected intraperitoneally with 100 μg of neutralising antibody or isotype control on day 7 or day 6, 8, and 10. Anti-IL-17 mAb MAB421 (IgG2a) was purchased from R&D Systems (Minneapolis, MN, USA). IgG2a isotype control was purchased from eBioscience (San Diego, CA, USA).

Analysis of cytokine production

Mice were sacrificed on the indicated day. Spleens were harvested and haemolysed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$ and 0.004% $EDTA_2Na$ in PBS. Single-cell suspensions were prepared in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FCS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 50 μM 2-mercaptoethanol. CD4+ T cells were isolated by MACS positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the collected cells (>97%) was confirmed by flow cytometry. Splenic feeder cells treated with 50 μg/ml of mitomycin C were used as antigen presenting cells (APCs). The purified CD4+ T cells and APCs were co-cultured with 10 μM of the synthetic peptide at a ratio of 5:1 at 37°C under 5% CO_2 for 24 hours. The supernatants were assayed for interferon (IFN)-γ and IL-17 by Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Intracellular cytokine staining and flow cytometric analysis

Mice were sacrificed on day 5. The draining lymph nodes were harvested and single cell suspensions were prepared as described above. Cells (1×10^6/ml) were stimulated with 10 μM of the synthetic peptides in 96-well round bottom plates (Nunc, Roskilde, Denmark) for 24 hours and GoldiStop (BD PharMingen, San Diego, CA) was added for the last four hours of each culture. Cells were first stained extracellularly, fixed and permeabilised with Cytofix/Cytoperm solution (BD PharMingen, San Diego, CA) and then stained intracellularly.

Samples were acquired on FACSCalibur (BD PharMingen, San Diego, CA) and data were analysed with FlowJo (Tree Star, Ashland, OR).

Analysis of anti-GPI antibody

Sera were taken from immunised mice on day 14 and diluted 1:500 in blocking solution (25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) in PBS) for antibody analysis. We also prepared 96-well plates (Sumitomo Bakelite, Tokyo, Japan) coated with 5 μg/ml rhGPI or recombinant mouse GPI for 12 hours at 4°C. After washing twice with a washing buffer (0.05% Tween20 in PBS), the blocking solution was used for blocking nonspecific binding for two hours at room temperature. After three washes, 150 μl of the diluted serum was added and incubated for two hoursat room temperature. After three washes, alkaline phosphatase-conjugated anti-mouse IgG was added at a final dilution of 1:5000, for one hour at room temperature. After three washes, colour was developed with substrate solution (1 alkaline phosphatase tablet (Sigma-Aldrich, St. Louis, MO, USA) per 5 ml alkaline phosphatase reaction solution (containing 9.6% diethanolamine and 0.25 mM $MgCl_2$, pH 9.8)). Plates were incubated for 20 minutes at room temperature and optical density was measured by a microplate reader at 405 nm.

Immunohistology

For immunohistology, cryostat sections from ankle joints were prepared with the tape capture technique as described previously [8]. Briefly, ankle joints were taken from immunised mice on day 14 and placed in Tissue-Tek (Sakura Finetek, Torrance, CA) filled with 4% carboxymethyl cellulose compound (Finetec, Tokyo, Japan). Frozen ankles joints in the carboxymethyl cellulose compound were attached to the adhesive Cryofilm (Finetec, Tokyo, Japan) and were cut in the microtome. The sections on the adhesive film were fixed with cold acetone. After blocking with 2% bovine serum albumin and 0.05% Tween in PBS, the sections were stained with Alexa 546-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) (200 ng/slide), and nuclei were counterstained with 4',6-diamidino-2-phenyindole dilactate (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) (50 ng/slide). Fluorescence was detected with the Leica DMRA2 microscopy (Leica, Wetzlar, Germany). The images were acquired and processed with Leica FW4000 (Leica, Wetzlar, Germany).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). Differences between groups and variables were examined for statistical significance using the Mann-Whitney's U test and the Spearman's rank correlation coefficient, respectively. A $p < 0.05$ denoted the presence of a statistically significant difference.

Results

I-A^q binding motif and epitope candidates

To analyse T cell epitopes, we first investigated the binding motif of I-A^q from T cell epitopes reported in the literature because DBA/1 mice express only I-A^q as MHC class II. Based on the work by Bayrak and colleagues [9], the anchor motif of I-A^q would exist at P1, P4 and P7, therefore we predicted the binding motifs from amino acid sequences of I-A^q restricted epitopes on murine $RNase_{90-105}$ [10], myelin basic $protein_{89-101}$ [11,12], chicken type II collagen $(CII)_{181-209}$ [13], rat $CII_{256-270}$ [14,15], bovine $CII_{256-270}$ [4] and mouse type II collagen [9] (Table 1). Next, we selected 32 types of peptides as core sequences from the human GPI 558 amino acid sequence, which is thought to bind the binding motif (Table 2), and synthesised 25 kinds of 20-mer peptides for screening, each containing the core sequence in its centre (Table 3).

Epitope screening

rhGPI-specific CD4$^+$ T cells differentiate into Th1 and Th17 [2], so we analysed IFN-γ and IL-17 production for epitope screening when rhGPI-primed CD4$^+$ T cells were stimulated with each synthetic peptide. The production of both IFN-γ and IL-17 was pronounced when GPI-primed CD4$^+$ T cells were stimulated with number 18 peptide ($hGPI_{327-346}$) and number 25 peptide ($hGPI_{539-558}$). Therefore, we considered that major epitopes exist in either of the two peptides (Figure 1). In the K/BxN mouse model of arthritis, KRN T cell receptor (TCR) transgenic T cells recognise $mGPI_{282-294}$, the dominant epitope of K/BxN mouse, on I-A^{g7} [16]. However, in the GPI-induced arthritis model, it was unlikely that $hGPI_{282-294}$ was the dominant epitope because GPI-specific T cells did not react prominently to number 16 peptide ($hGPI_{280-299}$).

Because the synthetic peptides used for screening were not purified, we re-synthesised the 15-mer peptides with a purity of 90%; these peptides contained each core sequence of

Table 1

I-Aq binding motifs

P1	P2	P3	P4	P5	P6	P7	P8	P9
A			A			E		
F			P			D		
L			F			Q		
I			S			P		
P			V			N		
S			L			I		
V			N					
			R					

The anchor motif of I-A^q would exist at P1, P4 and P7, therefore we predicted the binding motif from amino acid sequences of I-A^q restricted epitopes on murine $RNase_{90-105}$, myelin basic $protein_{89-101}$, chicken type II $collagen_{181-209}$, rat type II $collagen_{256-270}$, bovine type II $collagen_{256-270}$ and mouse type II collagen.

Table 2

Core sequences of glucose-6-phosphate isomerase (GPI) amino acids binding I-A^q

Peptide	Amino acid residues
3–11	ALTRDPQFQ
29–37	LFDANKDRF
41–49	SLTLNTNHG
56–64	SKNLVTEDV
72–80	AKSRGVEAA
80–88	ARERMFNGE
99–107	LHVALRNRS
102–110	ALRNRSNTP
149–157	ITDVINIGI
167–175	VTEALKPYS
173–181	PYSSGGPRV
181–189	VWYVSNIDG
196–204	LAQLNPESS
201–209	PESSLFIIA
210–218	SKTFTTQET
229–237	FLQAAKDPS
230–238	LQAAKDPSA
243–251	FVALSTNTT
253–261	VKEFGIDPQ
285–293	ALHVGFDNF
319–327	LLALLGIWY
328–336	INCFGCETH
337–345	AMLPYDQYL
391–399	FYQLIHQGT
403–411	PCDFLIPVQ
407–415	LIPVQTQHP
426–434	LANFLAQTE
452–460	AGKSPEDLE
489–497	ALVAMYEHK
537–545	SHDASTNGL
540–548	ASTNGLINF
545–553	LINFIKQQR

Thirty-two types of peptides were selected as core sequences from the GPI 558 amino acid sequence, which is thought to bind the binding motif. Amino acid residues that are thought to bind anchors of I-A^q are shown in bold letters.

number 18 peptide ($hGPI_{327-346}$) and number 25 peptide ($hGPI_{539-558}$). Number 18 peptide ($hGPI_{327-346}$) contains two core sequences ($hGPI_{328-336}$ and $hGPI_{337-345}$), so therefore we re-synthesised two peptides ($hGPI_{325-339}$ and $hGPI_{334-348}$). The former sequences of number 25 peptide ($hGPI_{539-558}$) overlapped with number 24 peptide ($hGPI_{533-552}$), which could not stimulate CD4+ T cells primed with GPI. Therefore we re-synthesised two peptides ($hGPI_{542-556}$ and $hGPI_{544-558}$) from the latter sequences of number 25 peptide (Table 4). We analysed IFN-γ and IL-17 production for epitope screening as described above. The peptide ($hGPI_{325-339}$) induced marked stimulation of GPI-primed CD4+ T cells, and was considered a major epitope (Figure 2).

Immunisation with a major epitope induces arthritis similar to GPI-induced arthritis

To test if $hGPI_{325-339}$ is arthritogenic, DBA/1 mice were immunised with 10 μg or 25 μg $hGPI_{325-339}$ instead of GPI protein, and 200 ng of pertussis toxin was injected intraperitoneally on days 0 and 2 after immunisation. Arthritis resembling GPI-induced arthritis could be generated by immunisation with the peptide, including incidence, manifestations and severity. Symmetrical polyarthritis appeared on day 8, showed peak severity on day 14 and subsided gradually thereafter (Figure 3a). The use of different immunisation doses (10 and 25 μg) did not seem to affect the incidence and severity of arthritis. Immunised with 10 μg or 25 μg $hGPI_{325-339}$ without injection of pertussis toxin could also induce arthritis. However, the arthritis was less severe than with pertussis toxin (data not shown). On the other hand, immunisation with neither $hGPI_{539-558}$ nor $hGPI_{544-558}$, which were considered minor epitopes in GPI-induced arthritis, could induce overt arthritis (Figure 3a). Mice immunised with $hGPI_{325-339}$ developed severe swelling of the wrist and ankle joints. Histologically, severe synovitis was noted in the wrists in the forepaws, and at ankles and tarsal joints in the hind paws (Figure 3b and data not shown).

Peptide-induced arthritis is mediated by Th17

GPI-induced arthritis is Th17-mediated [2], so we explored the aetiological role of Th17 in peptide-induced arthritis. Like GPI-induced arthritis, one time administration of anti-IL-17 mAb on day 7 and three times administration on day 6, 8 and 10 significantly ameliorated the arthritis (Figure 4). From these data, the arthritis induced by $hGPI_{325-339}$ was also considered to be Th17 mediated.

Immunisation of human $GPI_{325-339}$ leads Th17 cells to cross-react with mouse $GPI_{325-339}$

We examined the pathogenesis of arthritis induced by $hGPI_{325-339}$ by comparing it with mice immunised with $hGPI_{544-558}$.

First, we speculated that the difference in cross-reactivity to mouse GPI might affect the incidence of arthritis, because $hGPI_{325-339}$ (IWYINCFGCETHAML) has 13/15 amino acids homology to $mGPI_{325-339}$ (IWYINCYGCETHALL) while $hGPI_{544-558}$ (GLINFIKQQREARVQ) has only 9/15 amino