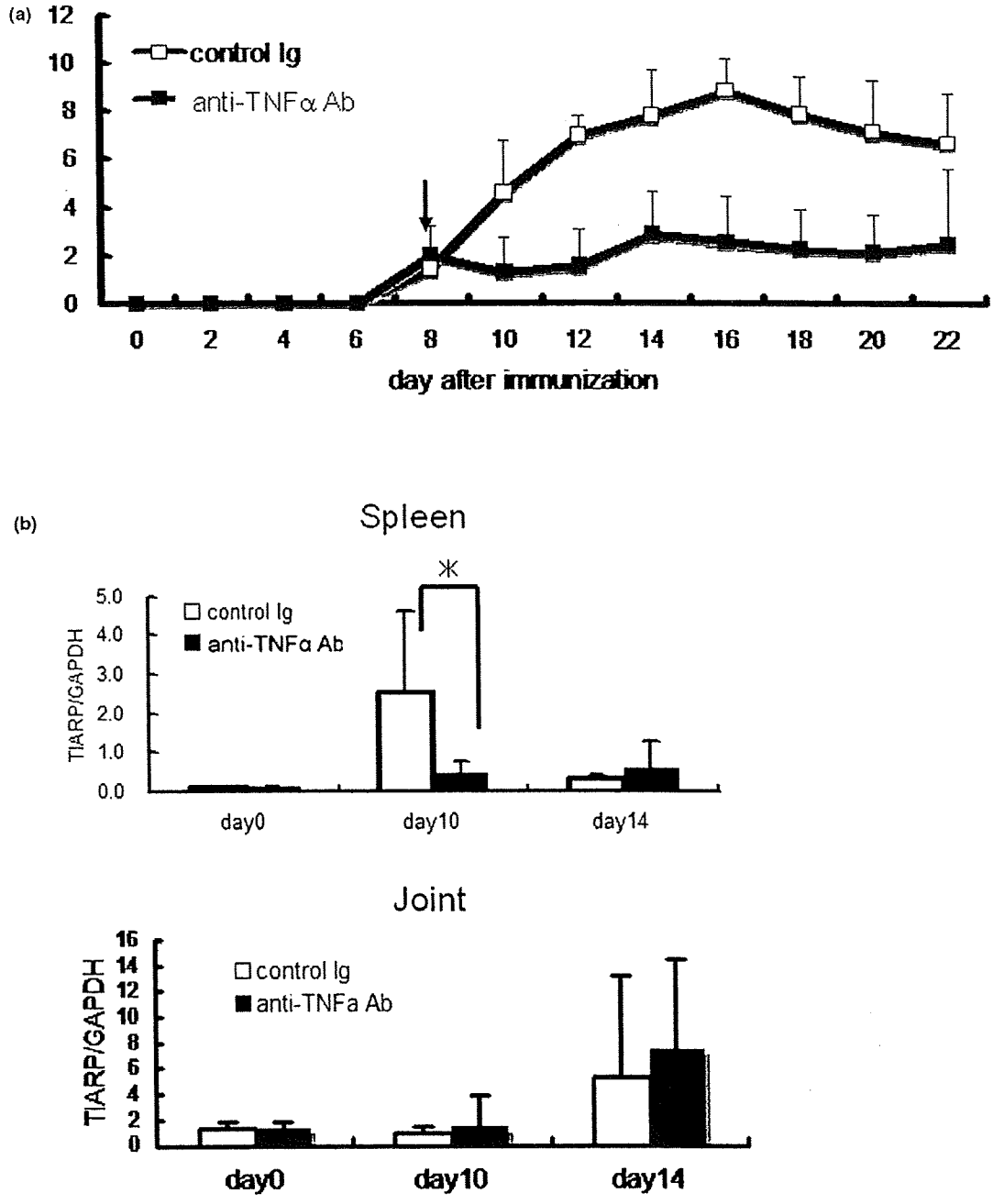
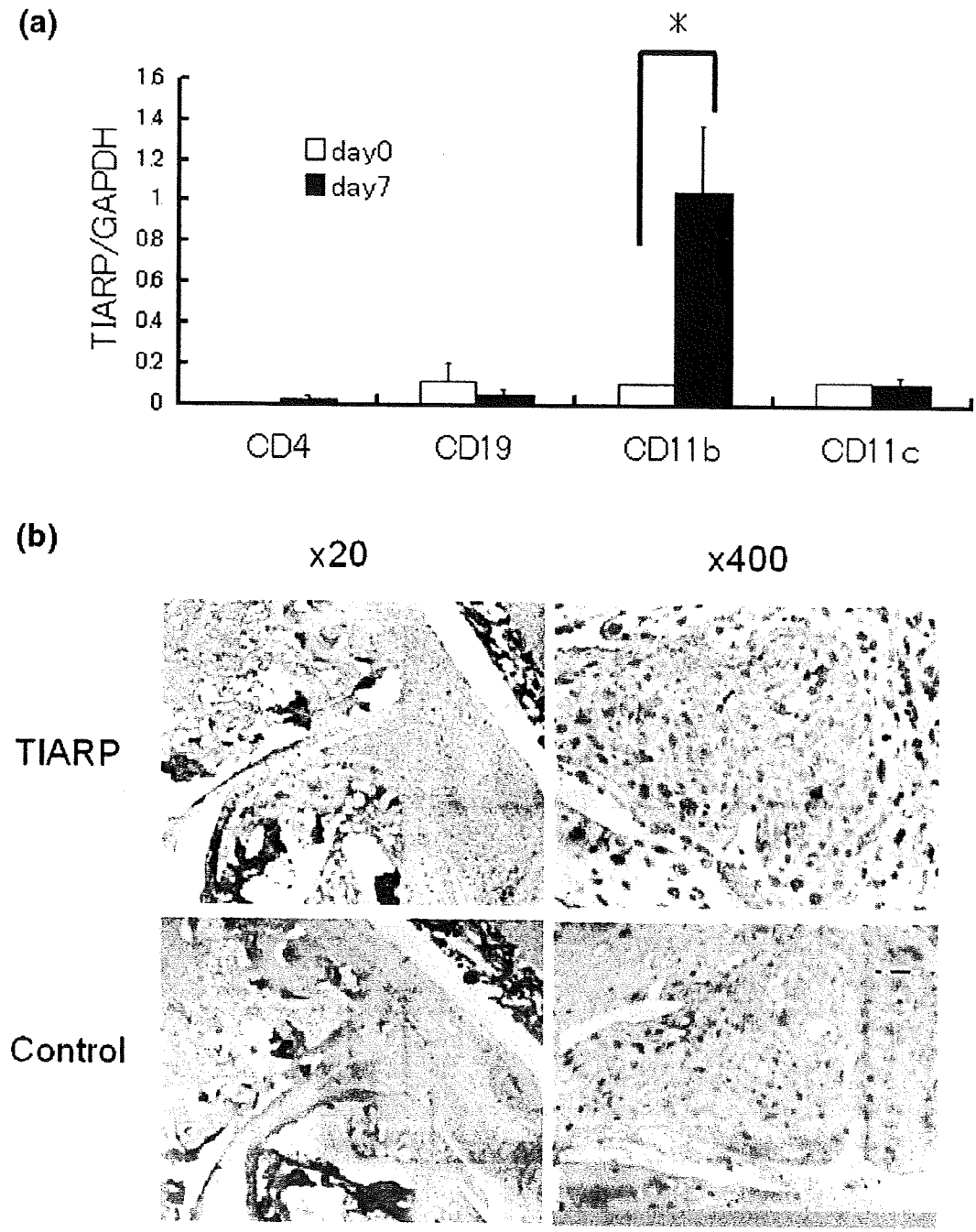
Figure 3



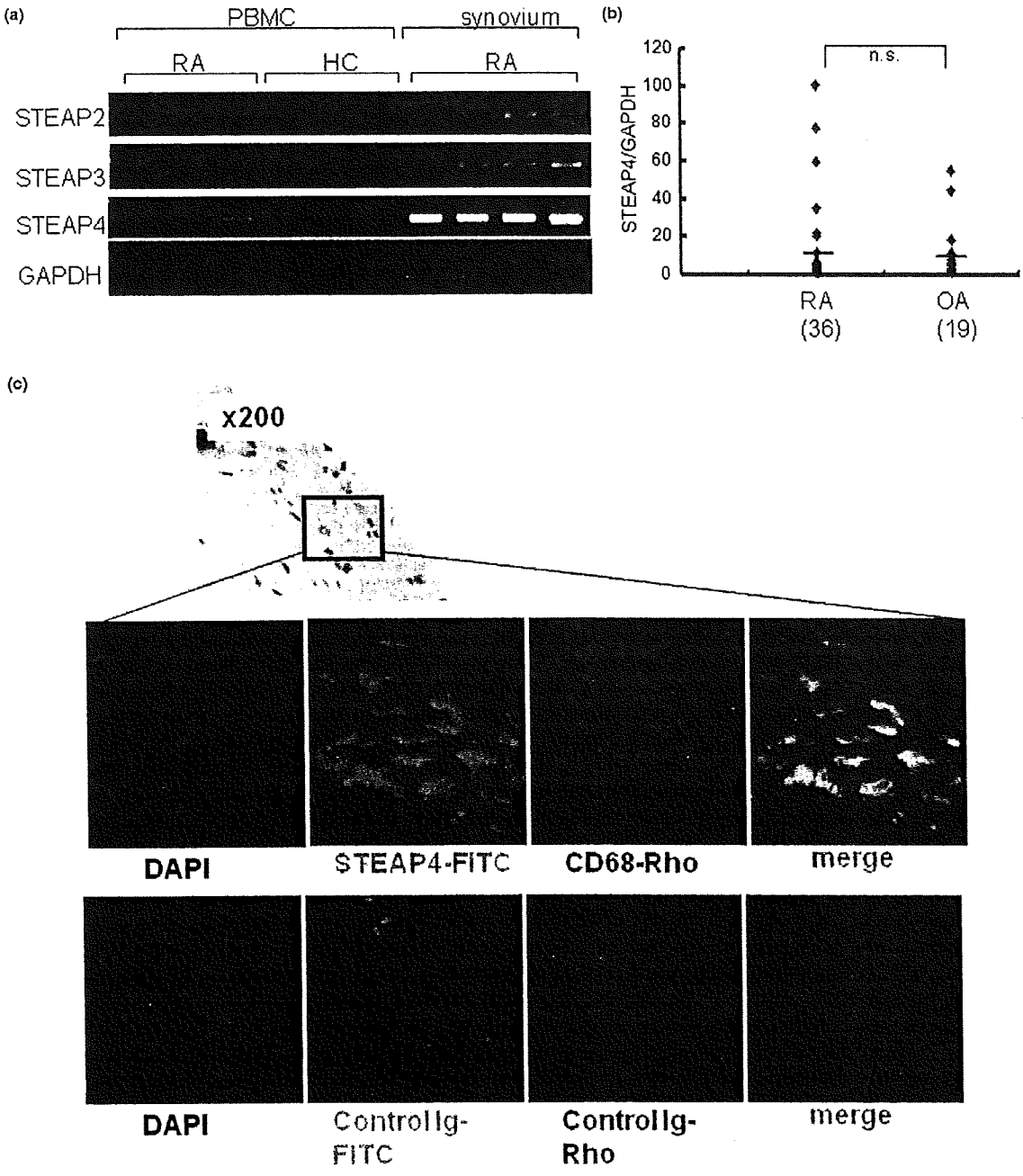
Suppression of TIARP mRNA by treatment with anti-tumor necrosis factor-alpha monoclonal antibody (anti-TNFα mAb). **(a)** The development of arthritis was blocked by administration of anti-TNFα mAb in mice immunized with glucose-6-phosphate isomerase. Data represent arthritis scores. **(b)** In spleen, 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

Y. Tanaka-Watanabe,* I. Matsumoto,*† K. Iwanami,* A. Inoue,* D. Goto,* S. Ito,* A. Tsutsumi* and T. Sumida*
*Division of Clinical Immunology, Major of Advanced Biomedical Applications, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai, Tsukuba, and †PRESTO, Japan Science and Technology Agency, Honcho Kawaguchi, Saitama, Japan

Accepted for publication 8 October 2008
Correspondence: I. Matsumoto, Division of Clinical Immunology, Advanced Biomedical Applications, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan.
E-mail: ismatsu@md.tsukuba.ac.jp

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, FcγR$^{-/-}$ 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

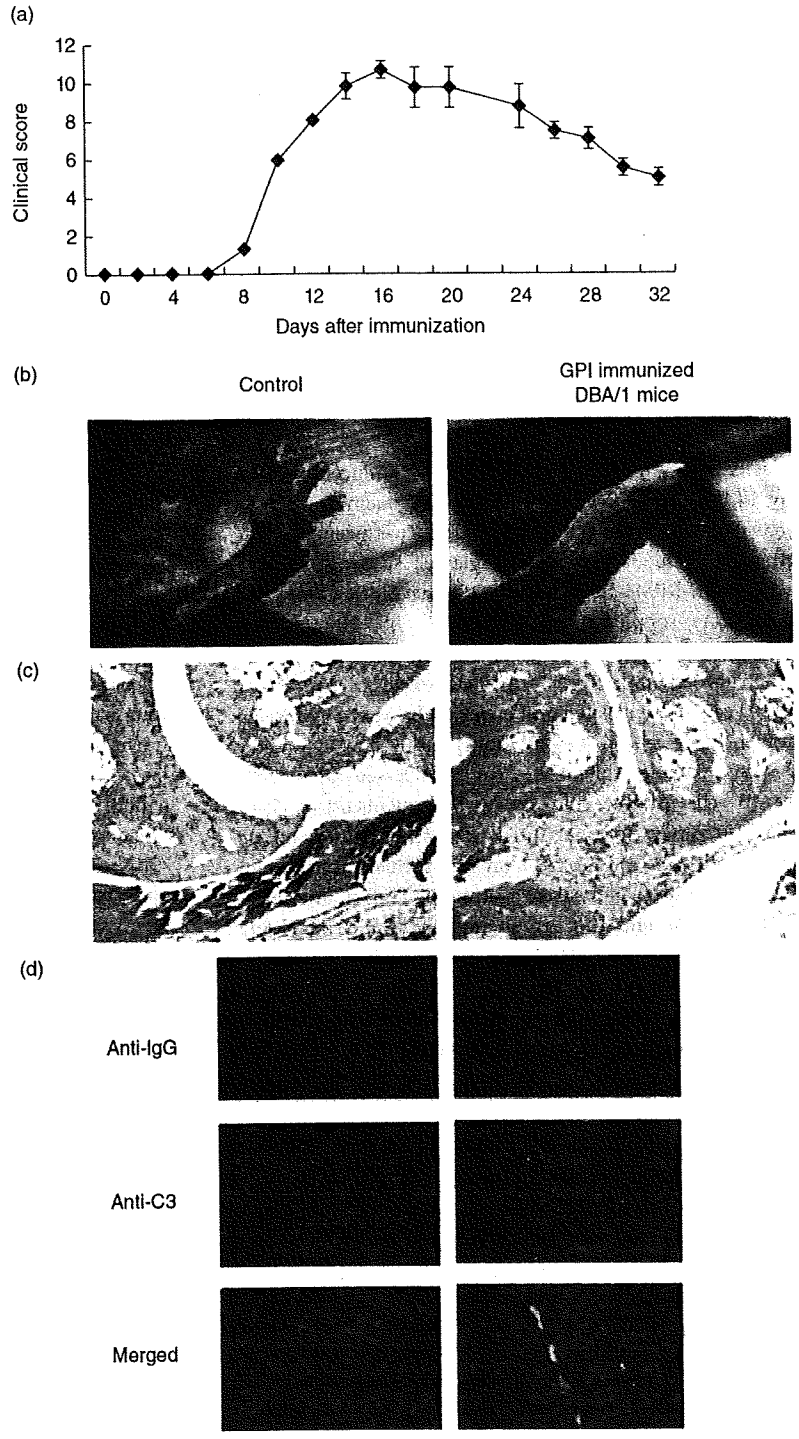

Fig. 1. Clinical and histological evaluation of glucose-6-phosphate isomerase (GPI)-induced arthritis in DBA/1 mice. Mean of clinical score (a) (±standard error of the mean, 10 mice) followed days after immunization. (b) Paw of control DBA/1 mouse treated with control antigens [gluthathione S-transferase (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

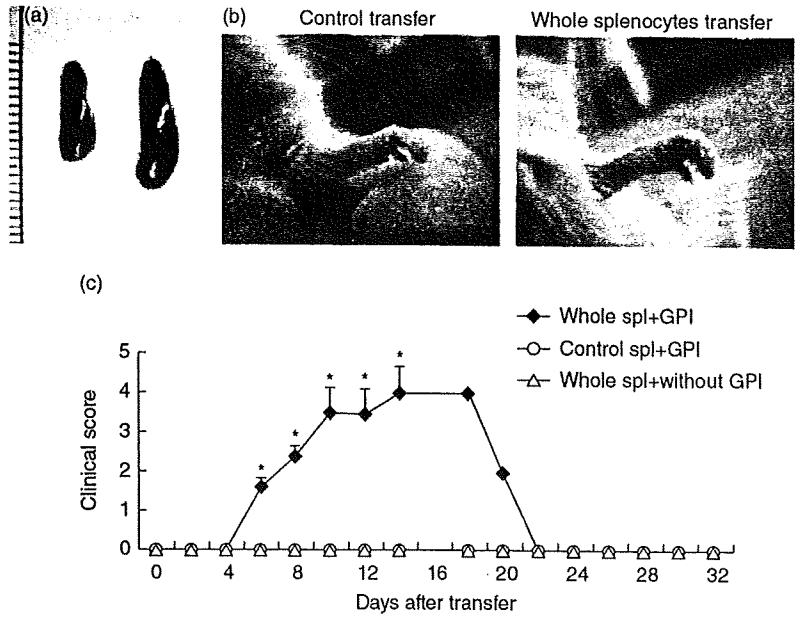

Fig. 2. Transfer of arthritis in SCID mice. Glucose-6-phosphate isomerase (GPI)-immunized DBA/1 mice were killed on day 14, and 1×10^7 splenocytes (spl) were isolated and transferred into SCID mice with 100 μg of GPI. (a) Spleen of control SCID mice (left) and SCID mice inoculated with splenocytes of 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 1 mg 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

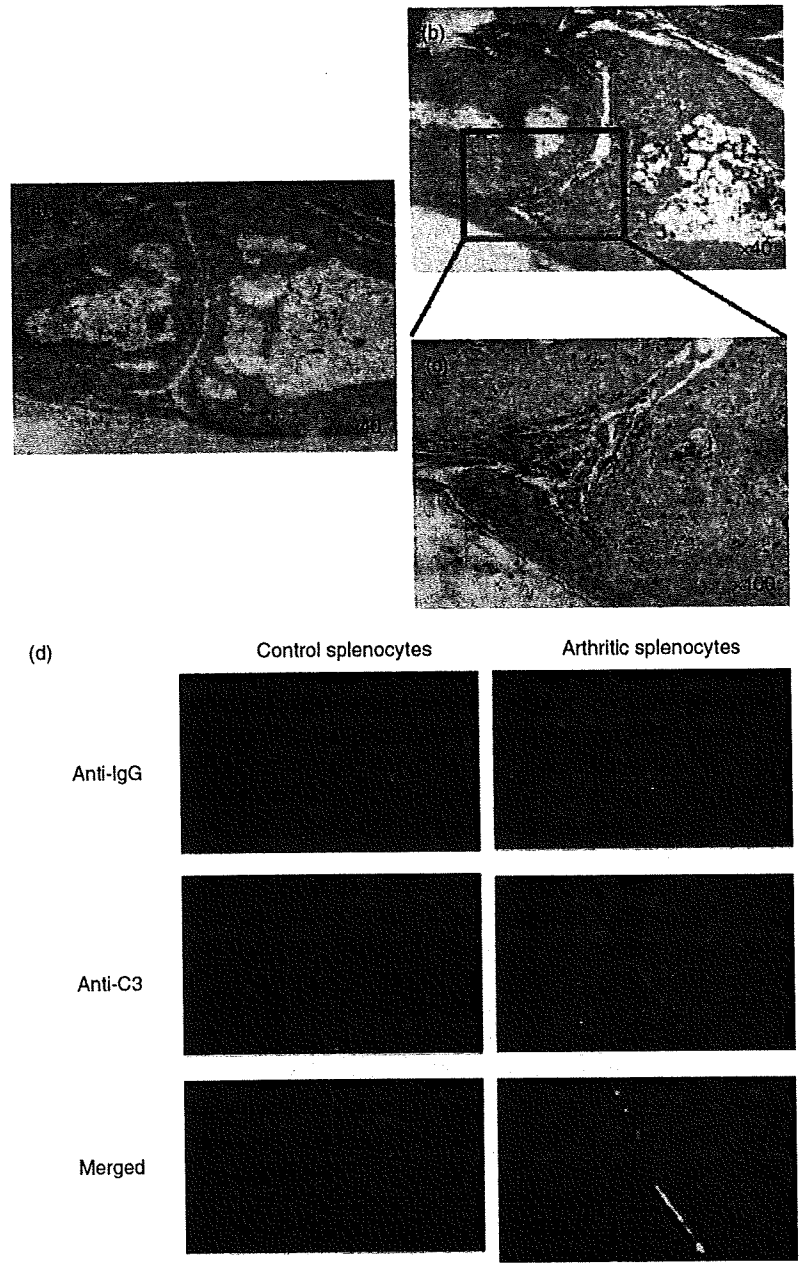

Fig. 3. Histological evaluation of joints of SCID mice. Joints of control mice inoculated splenocyte (spl) from naive DBA/1 mice with 100 μg of glucose-6-phosphate isomerase (GPI). (b,c) Synovial hyperplasia in a representative SCID mouse inoculated with splenocytes from arthritic DBA/1 mice. (d, right) 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.

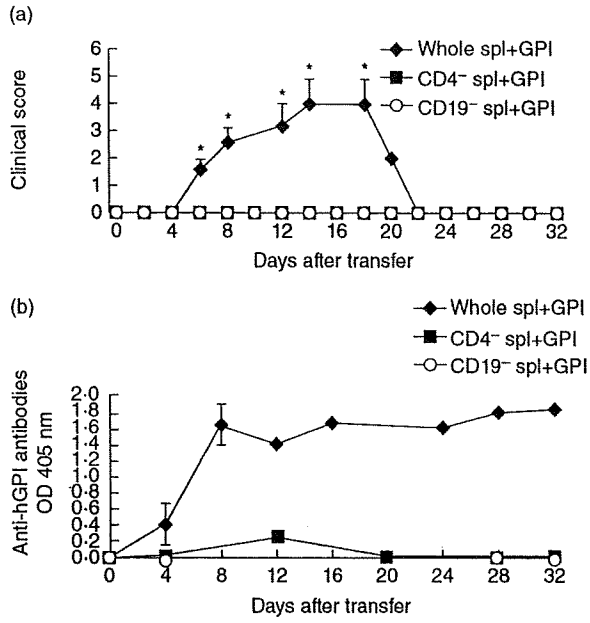

Fig. 4. Importance of anti-GPI antibodies in transfer of arthritis. CD19-depleted or CD4⁺-depleted splenocytes (spl) from arthritic DBA/1 mice obtained on day 14 after immunization were inoculated with glucose-6-phosphate isomerase (GPI) into SCID mice. (a) Mean clinical score. (b) Anti-GPI antibodies 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. *$P < 0.05$ 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 ($P < 0.05$), 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

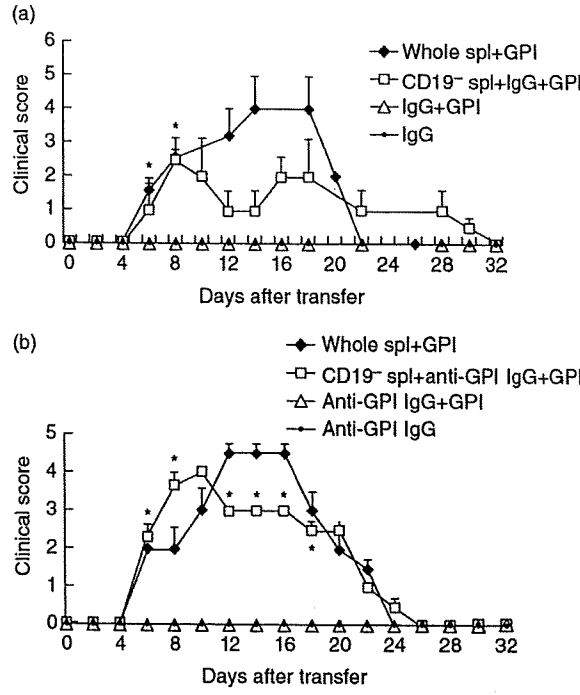

Fig. 5. Role of B cells in induction of arthritis in SCID mice. IgG from arthritic DBA/1 mice [alone or with glucose-6-phosphate isomerase (GPI)] or immunoglobulin (Ig)G plus CD19⁺-depleted cells were inoculated into SCID mice. Development of arthritis in SCID mice was monitored. (a) Mean clinical score 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. *$P < 0.05$ by Mann–Whitney *U*-test.

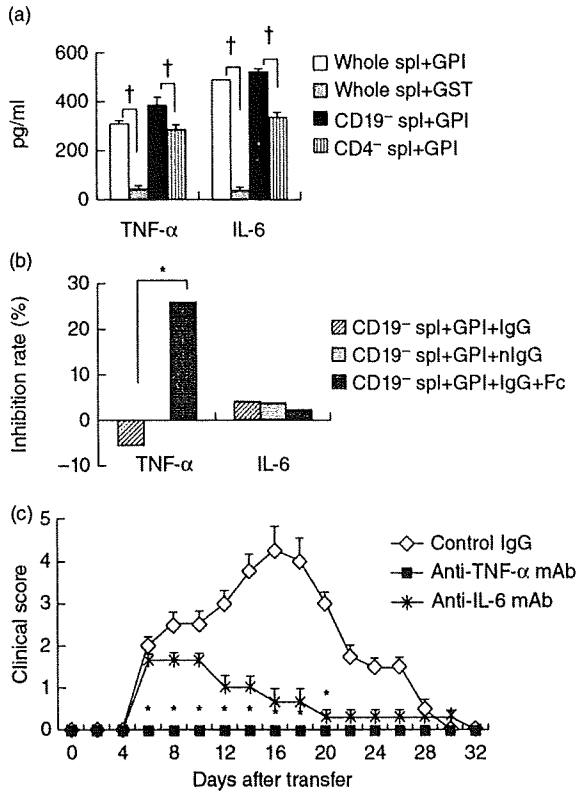

Fig. 6. *In vitro* cytokine production by splenocytes from arthritic DBA/1 mice and *in vivo* neutralization of inflammatory cytokines in SCID mice. Cytokine concentrations in supernatant of cultured splenocytes (spl) from arthritic DBA/1 mice were assessed by enzyme-linked immunosorbent assay. (a) Whole 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. $^*P < 0.05$, $^\dagger P < 0.01$, 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.

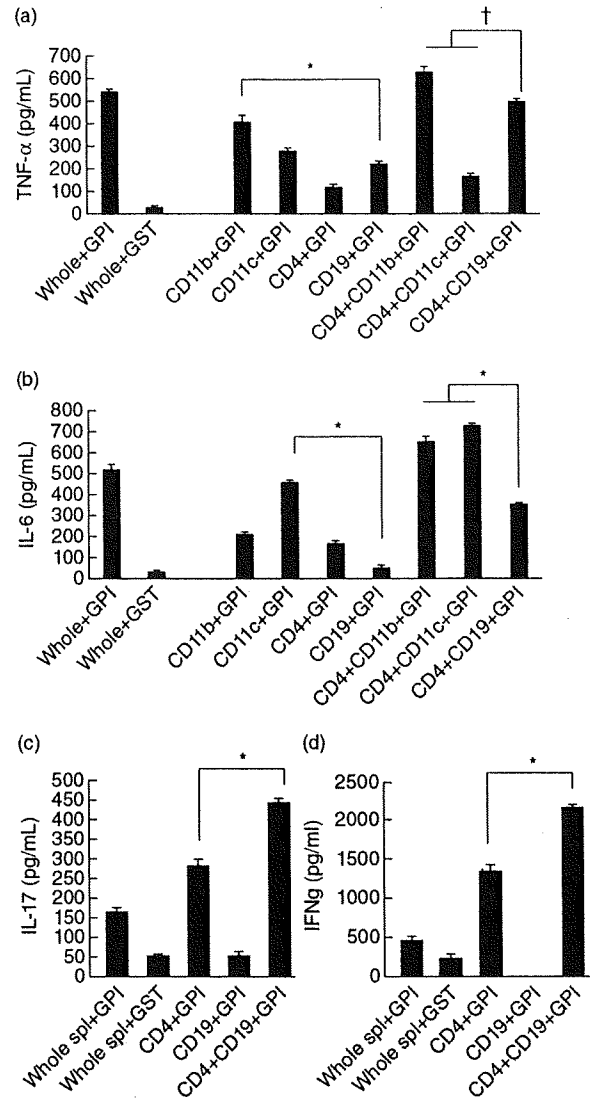

Fig. 7. Exploration of dominant cell population of inflammatory cytokines, and B cell functions as an antigen-presenting cells (APCs) *in vitro*. Whole splenocytes (spl) or independent magnetic affinity cell sorting (MACS) separated ($CD4^+$, $CD19^+$, $CD11b^+$ and $CD11c^+$ cells) splenocytes (total 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. $^*P < 0.05$, $^\dagger P < 0.001$, 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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Replication of the Association Between the *C8orf13–BLK* Region and Systemic Lupus Erythematosus in a Japanese Population

Ikue Ito,[1] Aya Kawasaki,[1] Satoshi Ito,[1] Taichi Hayashi,[1] Daisuke Goto,[1] Isao Matsumoto,[1] Akito Tsutsumi,[1] Geoffrey Hom,[2] Robert R. Graham,[2] Yoshinari Takasaki,[3] Hiroshi Hashimoto,[3] Jun Ohashi,[4] Timothy W. Behrens,[2] Takayuki Sumida,[1] and Naoyuki Tsuchiya[1]

Objective. **Recent genome-wide association studies identified an association between single-nucleotide polymorphisms (SNPs) in the *C8orf13* region of *BLK*, the B lymphoid tyrosine kinase gene, with systemic lupus erythematosus (SLE) in Caucasians. The purpose of this study was to evaluate the significance of this region in the genetic background of Japanese patients with SLE.**

Methods. **Fourteen tag SNPs in the *C8orf13–BLK* region were genotyped in 327 Japanese patients with SLE and 322 healthy Japanese controls. The population-attributable risk percentage (PAR%) of rs13277113 in Japanese was compared with that in Caucasians as well as with that of other SLE susceptibility genes in Japanese.**

Results. **As in Caucasians, rs13277113A demonstrated the strongest association in Japanese ($P = 1.73 \times 10^{-6}$ for the genotype frequency, $P = 4.75 \times 10^{-7}$ for the allele frequency, odds ratio [OR] 2.44 [95% confidence interval (95% CI) 1.43–4.16]). The association in Japanese was consistent with a recessive model ($P = 2.74 \times 10^{-7}$, OR 2.27 [95% CI 1.66–3.11]). In contrast to the Caucasian population, this risk allele was the major allele in the Japanese population. Because both the risk allele frequency and the OR were higher in Japanese than in Caucasians, the PAR% of rs13277113 was estimated to be much higher in Japanese (35.4%) than in Caucasians (16.2%), and the second highest among the 6 confirmed SLE susceptibility genes in Japanese.**

Conclusion. **The association of the *C8orf13–BLK* region with SLE was replicated in a Japanese population. Contribution of this region to the genetic predisposition to SLE appeared to be greater in Japanese than in Caucasians.**

Since it became widely used in 2006, the genome-wide association study platform has disclosed a number of susceptibility genes for complex diseases. Many such susceptibility genes have been replicated in independent studies not only of the same population, but also of different populations. Two recent studies provided convincing evidence that single-nucleotide polymorphisms (SNPs) in the *C8orf13–BLK* region of chromosome 8p23.1 are significantly associated with systemic lupus erythematosus (SLE; OMIM no. #152700) in Caucasians (1,2).

The *BLK* gene encodes a B lymphoid–specific tyrosine kinase of the Src family (3). B lineage cells express several distinct Src family protein kinases, such

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[1]Ikue Ito, Aya Kawasaki, PhD, Satoshi Ito, MD, PhD, Taichi Hayashi, MD, PhD, Daisuke Goto, MD, PhD, Isao Matsumoto, MD, PhD, Akito Tsutsumi, MD, PhD (current address: Takikawa Municipal Hospital, Takikawa, Japan), Takayuki Sumida, MD, PhD, Naoyuki Tsuchiya, MD, PhD: University of Tsukuba, Tsukuba, Japan; [2]Geoffrey Hom, PhD, Robert R. Graham, PhD, Timothy W. Behrens, MD: Genentech Inc., South San Francisco, California; [3]Yoshinari Takasaki, MD, PhD, Hiroshi Hashimoto, MD, PhD: Juntendo University, Tokyo, Japan; [4]Jun Ohashi, PhD: University of Tokyo, Tokyo, Japan.

Drs. Hom, Graham, and Behrens own stock or stock options in Genentech.

Address correspondence and reprint requests to Naoyuki Tsuchiya, MD, PhD, Doctoral Program in Life System Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: tsuchiya@md.tsukuba.ac.jp.

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as BLK, Fyn, and Lyn. These 3 are most strikingly expressed in B cells, in fact, *BLK* is the only tyrosine kinase of the Src family that is specifically expressed in B lineage cells (3). *C8orf13* encodes a ubiquitously expressed gene, the function of which remains unknown. The risk allele of the SLE-associated SNP rs13277113 has been shown to be associated with low levels of messenger RNA (mRNA) for *BLK* and high levels of mRNA for *C8orf13* (1). Thus far, the association of the *C8orf13–BLK* region has not been tested in non-Caucasian populations.

In the present study, we examined whether the association between the *C8orf13–BLK* region and SLE could be replicated in a Japanese population. In addition, we compared the contribution of this SNP to SLE in Japanese patients with that of other previously reported and replicated SLE susceptibility genes in Japanese by estimating their population-attributable risk percentage (PAR%).

PATIENTS AND METHODS

Study population. Three hundred twenty-seven Japanese patients with SLE (24 males and 303 females; mean ± SD age 41.6 ± 13.3 years) and 322 healthy control subjects (158 males and 164 females; mean ± SD age 30.6 ± 9.5 years) were studied. All patients fulfilled the American College of Rheumatology classification criteria for SLE (4). The patients and healthy controls were recruited at Tsukuba University Hospital, Juntendo University Hospital, and University of Tokyo Hospital. All patients and controls were unrelated Japanese. Genotype data for the Caucasian population were derived from our previously reported study (1). This study was reviewed and approved by the Research Ethics Committees of the University of Tsukuba, Juntendo University, and the University of Tokyo.

Preparation of genomic DNA. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp blood kit (Qiagen, Hilden, Germany) or a QuickGene-800 kit (Fujifilm, Tokyo, Japan). Whole-genome amplification was performed using a GenomiPhi DNA Amplification kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Genotyping. TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA) was used to determine the genotype of 14 tag SNPs in the *C8orf13–BLK* region. SNPs were selected based on the HapMap Phase II data for Japanese in Tokyo, Japan (JPT). From the JPT dataset, we chose SNPs with an r^2 threshold of 0.8 and minor allele frequency of ≥0.1. The genotype frequency data in Caucasian cases and controls were obtained from our previously reported study (1).

Statistical analysis. Association analyses were calculated by chi-square tests using 2 × 2 as well as 2 × 3 contingency tables. To find the best-fit model among the recessive, additive, and dominant models, a logistic regression analysis was performed for each SNP. Since under the recessive model, rs13277113 showed the lowest *P* value on logistic regression analysis, a conditional logistic regression analysis adjusted for rs13277113 (recessive model) was further conducted for each SNP to examine the effect on SLE susceptibility after controlling for rs13277113.

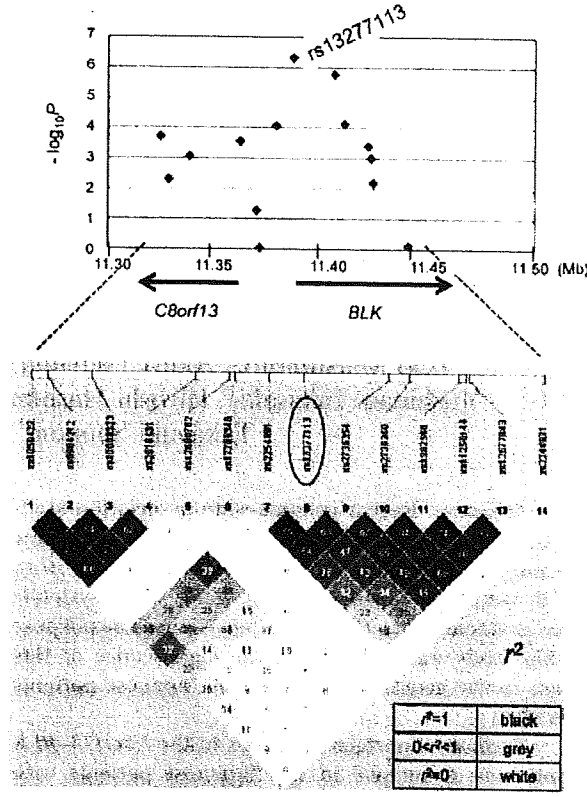

Figure 1. Linkage disequilibrium of 14 tag single-nucleotide polymorphisms (SNPs) in the *C8orf13–BLK* region in a Japanese population and allele associations with systemic lupus erythematosus. **Top,** Differences in the frequency of each of the 14 SNP alleles in the *C8orf13* and *BLK* regions. *P* values for differences in allele frequencies were calculated by chi-square test using 2 × 2 contingency tables; the $-\log_{10}$ *P* value for each SNP is shown. Arrows indicate the location and direction of transcription of *BLK* and *C8orf13*. **Bottom,** Correlation (r^2) values for each of the 14 SNPs based on data from 322 healthy Japanese control subjects. Haploview software version 4.0 (Broad Institute, Cambridge, MA; online at http://www.broadinstitute.com/haploview/haploview) was used to calculate the correlations.

The PAR% was estimated using the following formula:

$$PAR\% = Pe(RR - 1)/[Pe(RR - 1) + 1]$$

where Pe represents the risk genotype frequency in the population and RR represents relative risk of the risk genotype (5). Given the low prevalence of SLE, the Pe value can be estimated based on the genotype frequencies in healthy controls, and the RR value can be approximated by the odds ratio

Table 1. Allelic association of 14 tag SNPs in the *C8orf13–BLK* region in Japanese patients with systemic lupus erythematosus*

SNP	Chromosome position	Location	Risk allele	OR (95% CI)	*P*
rs10503423	11324491	*C8orf13* intron 2	C	1.40 (1.23–1.93)	1.93×10^{-4}
rs6984212	11328376	*C8orf13* intron 2	T	1.40 (1.11–1.77)	0.005
rs10088323	11338301	*C8orf13* intron 2	G	1.43 (1.18–1.87)	8.83×10^{-4}
rs2618431	11361874	*C8orf13* 5′ near gene	G	1.43 (1.22–1.95)	2.76×10^{-4}
rs12680762	11369436	Intergenic	T	1.41 (1.00–2.00)	0.053
rs17799348	11370931	Intergenic	T	1.01 (0.76–1.35)	0.925
rs2254891	11378539	Intergenic	C	1.66 (1.29–2.14)	8.37×10^{-5}
rs13277113	11386595	Intergenic	A	1.87 (1.47–2.40)	4.75×10^{-7}
rs2736354	11406140	*BLK* intron 1	C	1.83 (1.43–2.35)	1.67×10^{-6}
rs2736360	11410876	*BLK* intron 1	G	1.68 (1.30–2.17)	7.20×10^{-5}
rs1382566	11422250	*BLK* intron 1	G	1.57 (1.23–2.03)	3.95×10^{-4}
rs11250144	11423685	*BLK* intron 1	C	1.49 (1.17–1.88)	9.77×10^{-4}
rs12677843	11424598	*BLK* intron 1	T	1.38 (1.07–1.74)	0.006
rs2244931	11441178	*BLK* intron 3	C	1.04 (0.81–1.34)	0.762

* Odds ratios (ORs), 95% confidence intervals (95% CIs), and *P* values were calculated by chi-square test using 2 × 2 contingency tables based on allele frequencies. None of the genotypes in the controls showed significant deviation from Hardy-Weinberg equilibrium. Chromosome positions are according to the National Center for Biotechnology Information reference assembly. SNPs = single-nucleotide polymorphisms.

(OR) for each risk genotype. The PAR% for each susceptibility allele was estimated under the model (dominant or recessive) that provided the better fit, as revealed by the lower *P* value using the 2 × 2 contingency tables.

Although multiple comparisons should affect the interpretation of the statistical significance, because of the lack of a universally accepted method of correcting the *P* values for multiple SNPs in linkage disequilibrium and because our study involved the testing of a specific hypothesis, rather than being a hypothesis-generating study, we decided to present unadjusted *P* values and leave the interpretation to the reader.

RESULTS AND DISCUSSION

Fourteen tag SNPs in the *C8orf13–BLK* region were genotyped in 327 Japanese SLE patients and 322 healthy controls. Deviation from Hardy-Weinberg equilibrium was not observed for any of the SNPs in the control samples ($P = 0.134–0.955$). Figure 1 shows a linkage disequilibrium plot constructed from the Japanese control data.

Results of the case–control association analyses

Table 2. Results of logistic regression analysis of 14 SNPs for the development of systemic lupus erythematosus in Japanese, by analytical model

SNP	Correlation (r^2) with rs13277113*	Recessive model		Additive model		Dominant model	
		P†	*P* adjusted for rs13277113‡	*P*†	*P* adjusted for rs13277113‡	*P*†	*P* adjusted for rs13277113‡
rs10503423	0.326	0.002	0.445	0.0002	0.146	0.002	0.097
rs6984212	0.206	0.036	0.876	0.005	0.397	0.008	0.135
rs10088323	0.254	0.010	0.660	0.001	0.264	0.005	0.120
rs2618431	0.288	0.002	0.503	0.0004	0.223	0.005	0.162
rs12680762	0	0.042	0.064	0.053	0.081	0.008	0.799
rs17799348	0.002	0.963	0.618	0.926	0.743	0.692	0.771
rs2254891	0.763	8.85×10^{-6}	0.156	0.0002	0.461	0.078	0.858
rs13277113	NA	2.17×10^{-7}	NA	1.36×10^{-6}	NA	0.032	NA
rs2736354	0.63	1.34×10^{-7}	0.044	4.14×10^{-6}	0.211	0.157	0.808
rs2736360	0.475	1.49×10^{-5}	0.186	0.0001	0.412	0.266	0.703
rs1382566	0.434	3.27×10^{-5}	0.205	0.0006	0.657	0.616	0.283
rs11250144	0.345	0.001	0.367	0.001	0.481	0.109	0.969
rs12677843	0.313	0.004	0.580	0.007	0.854	0.245	0.662
rs2244931	0.011	0.806	0.690	0.762	0.954	0.186	0.312

* Pairwise linkage disequilibrium between rs13277113 and each of the other 13 single-nucleotide polymorphisms (SNPs) was determined by correlation (r^2) analysis of the controls. NA = not applicable.
† *P* values for each SNP under the recessive, additive, or dominant model were calculated by logistic regression analysis.
‡ *P* values were adjusted for rs13277113 under the recessive model, which provided the best fit. Under the recessive model, the association of all other SNPs lost significance, except for a marginal association of rs2736354 ($P = 0.044$).

Table 3. Estimated haplotype frequencies and results of association analyses in Japanese SLE patients and controls*

Haplotype	Allele							SLE patients	Control subjects	Permutated *P*
	rs2254891	rs13277113	rs2736354	rs2736360	rs1382566	rs11250144	rs12677843			
a	C	A	C	G	G	C	T	0.628	0.528	0.0026
b	G	G	T	A	C	G	C	0.138	0.199	0.026
c	C	A	C	G	G	G	C	0.057	0.046	0.993
d	G	G	T	G	G	C	T	0.023	0.042	0.384
g	C	G	T	A	C	G	C	0.012	0.037	0.029
e	G	G	C	G	G	C	T	0.033	0.032	1
f	C	A	T	A	C	G	C	0.028	0.031	1
h	C	A	C	G	G	C	C	0.029	0.017	0.792
i	C	A	C	G	C	G	C	0.015	0.010	0.995

* *P* values were calculated by permutation test (1,000,000 permutations) using Haploview software version 4.0 (Broad Institute, Cambridge, MA). SLE = systemic lupus erythematosus.

are shown in Table 1. Eleven of the 14 SNPs exhibited evidence of an association with SLE in our Japanese population ($P < 0.05$), among which SNP rs13277113 showed the lowest *P* value and the highest OR. Logistic regression analysis revealed that the recessive model provided the best fit for SNP rs13277113 (Table 2). SNP rs13277113 is located at the intergenic region of *BLK* and *C8orf13*, but in Japanese, it is contained in the haplotype block that includes *BLK*, but not *C8orf13*.

Most of the SNPs associated with SLE showed evidence of linkage disequilibrium with rs13277113 (Table 2 and Figure 1). However, conditional logistic regression analysis revealed that the association of all other SNPs lost statistical significance when conditioned on rs13277113 (Table 2), except for a marginal association of rs2736354 under the recessive model ($P = 0.044$).

Haplotype association analysis revealed that the major haplotype in Japanese (haplotype a) is the single risk haplotype for SLE and that it contains the rs13277113A allele (Table 3). Taken together, these results provided compelling evidence that, as in Caucasians, rs13277113A accounts for most, if not all, of the genetic effect of the *C8orf13–BLK* region in Japanese.

Table 4 shows the results of the association analysis of rs13277113 in Japanese patients with SLE as compared with that in Caucasian SLE patients. Some notable differences between the Caucasian and Japanese populations were observed. Risk allele A was the major allele in the Japanese control population, with an allele frequency of 0.652 in Japanese and 0.229 in Caucasians. In Japanese, the association was compatible with a recessive model, since the association in the heterozygotes did not reach statistical significance. On the other hand, in Caucasians, the dominant model provided a much lower *P* value, whereas under the recessive model, the OR was slightly higher. Strikingly,

Table 4. Association of rs13277113 with SLE in Japanese and Caucasian populations*

	Japanese population				Caucasian population			
	SLE patients (n = 327)	Control subjects (n = 322)	*P*	OR (95% CI)	SLE patients (n = 1,311)	Control subjects (n = 3,336)	*P*	OR (95% CI)
Genotype frequency								
A/A	207 (0.633)	139 (0.432)	1.73×10^{-6}	2.44 (1.43–4.16)	108 (0.082)	173 (0.051)	1.41×10^{-9}	1.90 (1.47–2.44)
A/G	95 (0.291)	142 (0.441)		1.10 (0.63–1.92)	551 (0.420)	1,183 (0.355)		1.41 (1.24–1.62)
G/G	25 (0.076)	41 (0.127)		Referent	652 (0.497)	1,980 (0.594)		Referent
Allele frequency								
A (vs. G)	509 (0.778)	420 (0.652)	4.75×10^{-7}	1.87 (1.47–2.39)	767 (0.293)	1,529 (0.229)	1.85×10^{-10}	1.39 (1.26–1.54)
Recessive model								
A/A (vs. A/G + G/G)	207 (0.633)	139 (0.432)	2.74×10^{-7}	2.27 (1.66–3.11)	108 (0.082)	173 (0.051)	8.55×10^{-5}	1.64 (1.28–2.10)
Dominant model								
A/A + A/G (vs. G/G)	302 (0.924)	281 (0.873)	0.032	1.76 (1.05–2.96)	659 (0.503)	1,356 (0.406)	2.60×10^{-9}	1.48 (1.30–1.68)

* *P* values, odds ratios (ORs), and 95% confidence intervals (95% CIs) were calculated by chi-square analysis using 2 × 3 (comparison of genotype frequencies) or 2 × 2 (other comparisons) contingency tables. Genotype data for the Caucasian population were obtained from our previously published study (1). SLE = systemic lupus erythematosus.

Table 5. PAR% values for the replicated systemic lupus erythematosus susceptibility genes*

Gene	Allele	Population	Model	Population frequency of risk genotype	OR of risk genotype	PAR%	Reference
C8orf13–BLK	rs13277113A	Japanese	Recessive	0.432	2.27	35.4	Present study
C8orf13–BLK	rs13277113A	Caucasian	Dominant	0.406	1.48	16.2	Present study
C8orf13–BLK	rs13277113A	Caucasian	Recessive	0.052	1.64	3.2	Present study
HLA-DRB1	DRB1*1501	Japanese	Dominant	0.124	2.97	19.6	6
FCGR2B	rs1050501C	Japanese	Recessive	0.053	2.19	5.9	8
TNFRSF1B (TNFR2)	rs60195947G	Japanese	Dominant	0.188	2.53	22.4	10
TNFSF13 (APRIL)	rs11552708G	Japanese	Dominant	0.803	2.01	44.7	12
IRF5	rs41298401C	Japanese	Recessive	0.652	1.55	26.4	14

* Population-attributable risk percentage (PAR%) values for the *C8orf13–BLK* rs13277113A allele in the Japanese and Caucasian populations were compared with the PAR% values for other systemic lupus erythematosus susceptibility alleles in the Japanese population. The model (dominant or recessive) that gave a lower *P* value by chi-square test using 2 × 2 contingency tables was selected for each allele. Because rs13277113A gave a lower *P* value under the dominant model but a slightly higher odds ratio (OR) under the recessive model in the Caucasian population, the PAR% values obtained under both models are shown.

the ORs were substantially higher in the Japanese population than in the Caucasian population.

A higher frequency of the risk genotype in the population and a higher OR result in a greater PAR%, which indicates a greater contribution of the genotype of interest in the population. Thus, the PAR% for SNP rs13277113 in the Japanese population was compared with that in the Caucasian population as well as with other SLE susceptibility genes in the Japanese population. For this purpose, we selected previously reported SLE susceptibility alleles in Japanese that have been replicated by 1 or more independent studies in Japanese or in East Asian populations, namely, *HLA-DRB1*1501* (6,7), *FCGR2B*-232Thr (rs1050501C) (8,9), *TNFRSF1B* (*TNFR2*)–196Arg (rs60195947G) (10,11), *TNFSF13* (*APRIL*)–67Gly (rs11552708G) (12,13), and *IRF5* (rs41298401C) (14,15). For each allele, the PAR% was estimated based on our previous data (6,8,10,12,14), under the dominant or recessive model that gave the better fit (lower *P* value by chi-square analysis using 2 × 2 contingency tables).

As shown in Table 5, the PAR% for rs13277113 in the Japanese population was much higher than that in the Caucasian population. This comparison may not be completely valid because the PAR% may be affected by the method of ascertainment of the patients. While all Japanese patients were ascertained at university hospitals, the Caucasian subjects consisted of multiple case–control series originally recruited for various independent studies; therefore, the method of ascertainment may vary slightly among the case–control sets (1). Nevertheless, the results strongly suggested that the contribution of rs13277113 to the development of SLE may play a greater role in Japanese than in Caucasians. In addition, the contribution of rs13277113 was the second highest among the tested susceptibility genes in the Japanese population.

This is the first replication study of the *C8orf13–BLK* SNP in a non-Caucasian population of SLE patients and controls. The findings in our Japanese population confirmed the previously reported findings in Caucasian populations (1,2) showing that the *C8orf13–BLK* region is associated with SLE. The higher frequency and OR of the risk genotype in our study population also indicated that the impact of this polymorphism is greater in Japanese SLE patients than in Caucasian SLE patients. Such information may help to identify future molecular targets for the development of new treatments.

AUTHOR CONTRIBUTIONS

Dr. Tsuchiya had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. I. Ito, Kawasaki, Behrens, Tsuchiya.

Acquisition of data. I. Ito, Kawasaki, S. Ito, Hayashi, Goto, Matsumoto, Tsutsumi, Takasaki, Hashimoto, Sumida, Tsuchiya.

Analysis and interpretation of data. I. Ito, Kawasaki, Hom, Graham, Behrens, Tsuchiya.

Manuscript preparation. I. Ito, Ohashi, Tsuchiya.

Statistical analysis. I. Ito, Hom, Ohashi.

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