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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	**A**LT**R**DP**Q**FQ
29–37	**L**FD**A**NK**D**RF
41–49	**S**LT**L**NT**N**HG
56–64	**S**KN**L**VT**E**DV
72–80	**A**KS**R**GV**E**AA
80–88	**A**RE**R**MF**N**GE
99–107	**L**HV**A**LR**N**RS
102–110	**A**LR**N**RS**N**TP
149–157	**I**TD**V**IN**I**GI
167–175	**V**TE**A**LK**P**YS
173–181	**P**YS**S**GG**P**RV
181–189	**V**WY**V**SN**I**DG
196–204	**L**AQ**L**NP**E**SS
201–209	**P**ES**S**LF**I**IA
210–218	**S**KT**F**TT**Q**ET
229–237	**F**LQ**A**AK**D**PS
230–238	**L**QA**A**KD**P**SA
243–251	**F**VA**L**ST**N**TT
253–261	**V**KE**F**GI**D**PQ
285–293	**A**LH**V**GF**D**NF
319–327	**L**LA**L**LG**I**WY
328–336	**I**NC**F**GC**E**TH
337–345	**A**ML**P**YD**Q**YL
391–399	**F**YQ**L**IH**Q**GT
403–411	**P**CD**F**LI**P**VQ
407–415	**L**IP**V**QT**Q**HP
426–434	**L**AN**F**LA**Q**TE
452–460	**A**GK**S**PE**D**LE
489–497	**A**LV**A**MY**E**HK
537–545	**S**HD**A**ST**N**GL
540–548	**A**ST**N**GL**I**NF
545–553	**L**IN**F**IK**Q**QR

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

Table 3

Synthetic peptides for screening T cell epitopes

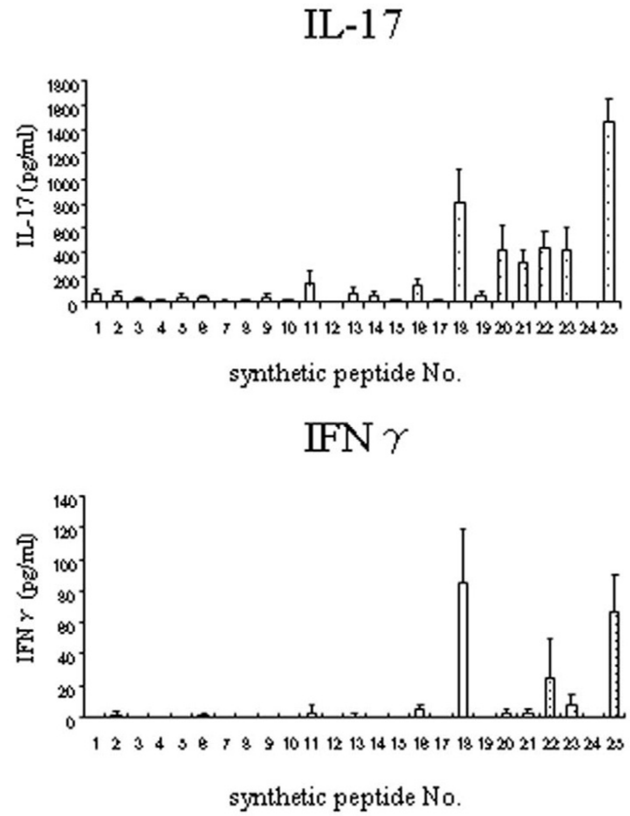
Peptide number	Peptide	Synthetic peptide sequence
1	1–20	H-MAALTRDPQFQKLQQWYREH-OH
2	23–42	H-ELNLRRLFDANKDRFNHFSL-OH
3	37–56	H-FNHFSLTLNTNHGHILVDYS-OH
4	51–70	H-ILVDYSKNLVTEDVMRMLVD-OH
5	71–90	H-LAKSRGVEAARERMFNGEKI-OH
6	96–115	H-RAVLHVALRNRSNTPILVDG-OH
7	145–164	H-TGKTITDVINIGIGGSDLGP-OH
8	162–181	H-LGPLMVTEALKPYSSGGPRV-OH
9	168–187	H-TEALKPYSSGGPRVWYVSNI-OH
10	176–195	H-SGGPRVWYVSNIDGTHIAKT-OH
11	191–210	H-HIAKTLAQLNPESSLFIIAS-OH
12	200–219	H-NPESSLFIIASKTFTTQETI-OH
13	225–244	H-AKEWFLQAAKDPSAVAKHFV-OH
14	238–257	H-AVAKHFVALSTNTTKVKEFG-OH
15	247–266	H-STNTTKVKEFGIDPQNMFEF-OH
16	280–299	H-IGLSIALHVGFDNFEQLLSG-OH
17	313–332	H-EKNAPVLLALLGIWYINCFG-OH
18	327–346	H-YINCFGCETHAMLPYDQYLH-OH
19	386–405	H-NGQHAFYQLIHQGTKMIPCD-OH
20	400–419	H-KMIPCDFLIPVQTQHPIRKG-OH
21	420–439	H-LHHKILLANFLAQTEALMRG-OH
22	445–464	H-ARKELQAAGKSPEDLERLLP-OH
23	484–503	H-PFMLGALVAMYEHKIFVQGI-OH
24	533–552	H-AQVTSHDASTNGLINFIKQQ-OH
25	539–558	H-DASTNGLINFIKQQREARVQ-OH

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

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

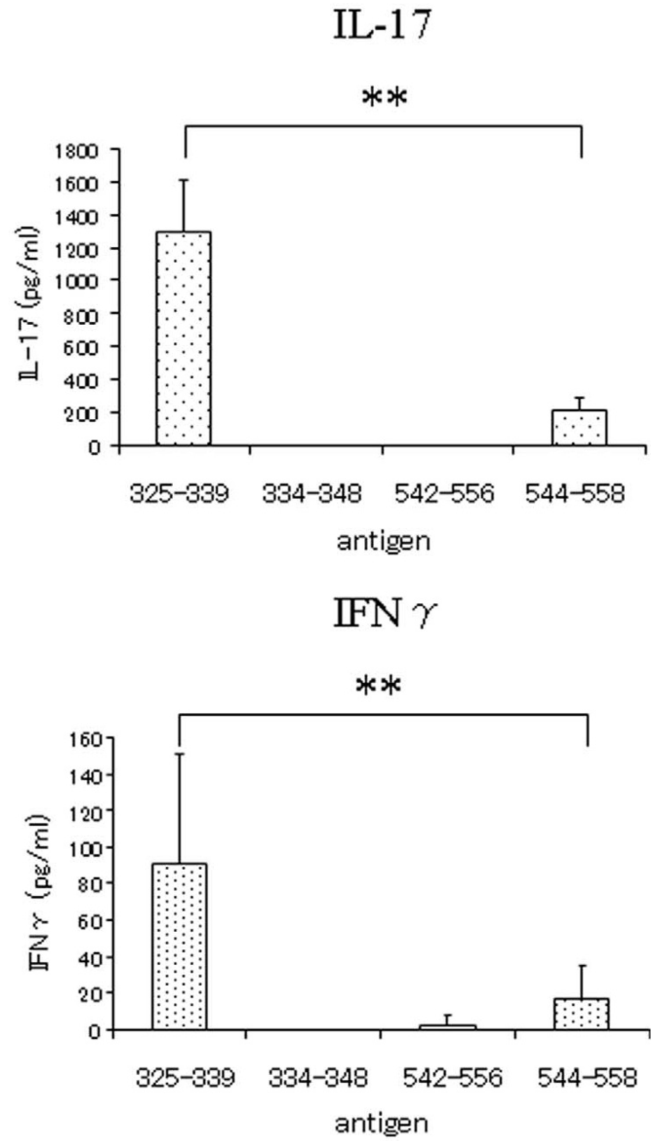
It has been reported that Th17 cells are not the only cellular sources of IL-17, but CD8$^+$ T cells, natural killer T cells and γδT cells are also capable of producing IL-17 [17-22]. Therefore, we investigated the IL-17 producing cells using flow cytometry. The draining lymph node cells from mice immunised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

Figure 1



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

Figure 2



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

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

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

To explore the importance of autoantibodies, we measured anti-human GPI antibodies and anti-mouse GPI antibodies in mice immunised with $hGPI_{325-339}$, $hGPI_{544-558}$ and $hGPI_{325-339}$ plus $hGPI_{544-558}$by ELISA. Mice immunised with rhGPI and the two peptides ($hGPI_{325-339}$ plus $hGPI_{544-558}$) produced high titres of anti-human GPI antibodies and anti-mouse GPI antibodies, and mice immunised with $hGPI_{325-339}$ and $hGPI_{544-558}$ hardly produced any anti-human GPI antibodies. However, mice immunised with $hGPI_{325-339}$ produced significantly higher titres of anti-mouse GPI antibodies than mice immunised with $hGPI_{544-558}$ (Figure 6a). It is noteworthy that immunisation with the two peptides ($hGPI_{325-339}$ plus $hGPI_{544-558}$) induced significantly higher titres of anti-mouse GPI antibodies than that with $hGPI_{325-339}$ alone, whereas the severity and incidence of arthritis in mice immunised with two peptides ($hGPI_{325-339}$ plus $hGPI_{544-558}$) were comparable with those in mice immunised with $hGPI_{325-339}$ alone (Figures 3a and 6a).

Table 4

Re-synthesised peptides used for determining a major epitope

Peptide number	Peptide	Synthetic peptide sequence
18	327–346	H-Y**I**NC**F**GC**E**TH**A**ML**P**YD**Q**YLH-OH
	325–339	H-IWY**I**NC**F**GC**E**THAML-OH
	334–348	H-ETH**A**ML**P**YD**Q**YLHRF-OH
25	539–558	H-DASTNG**L**IN**F**IK**Q**QREARVQ-OH
	542–556	H-TNG**L**IN**F**IK**Q**QREAR-OH
	544–558	H-G**L**IN**F**IK**Q**QREARVQ-OH

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

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

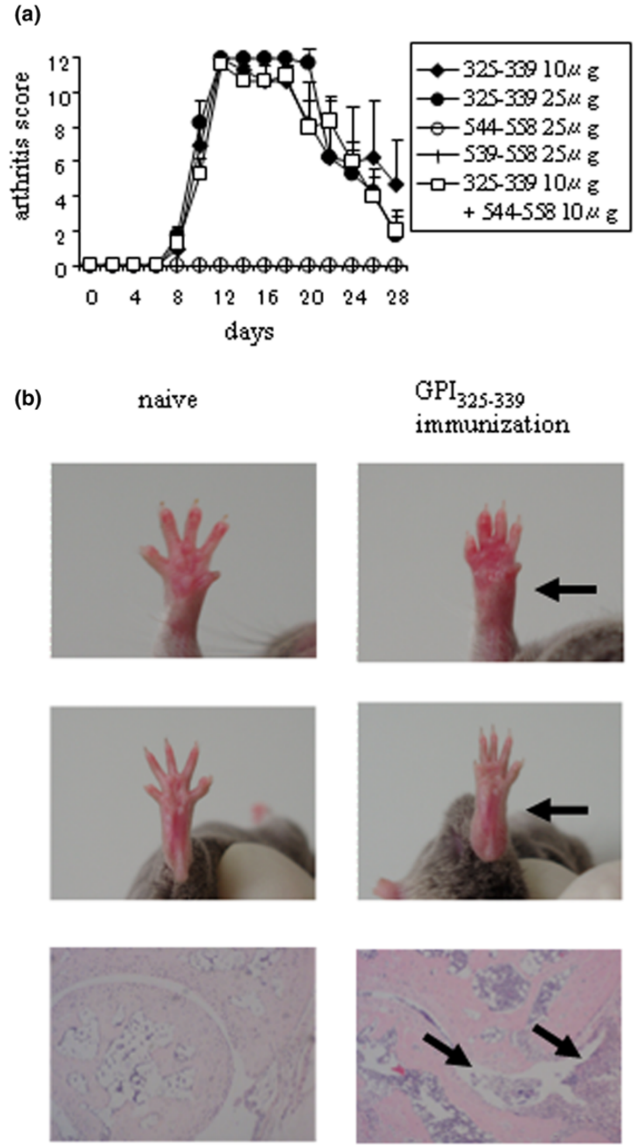
Next, we investigated the existence of IgG on the cartilage surface by immunohistology, because GPI were proved to deposit on the cartilage surface of normal naïve mice [23]. The cryostat sections of ankle joints from naïve mice and mice immunised with $hGPI_{544-558}$ did not show IgG deposit on the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

Discussion

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

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

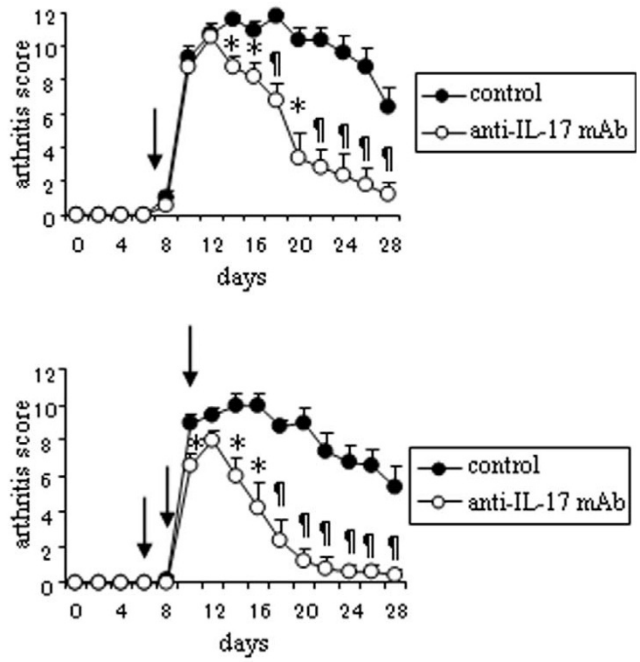
Figure 3



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

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

Figure 4



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

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

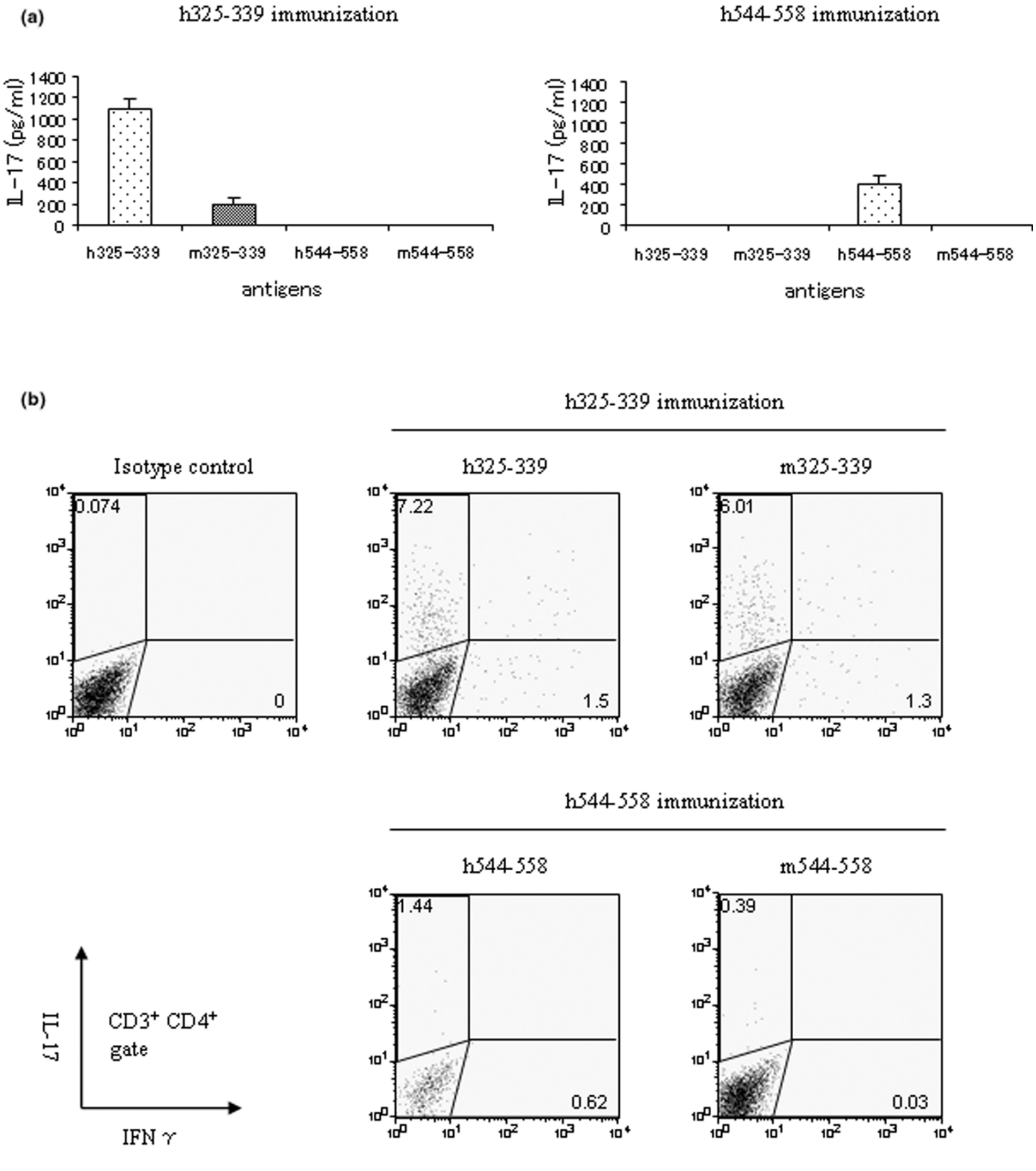
Our study demonstrated that immunisation with $hGPI_{325-339}$ induced antigen-specific Th17 cells, which can cross-react with $mGPI_{325-339}$ and lead B cells to produce anti-mouse GPI antibodies. However, immunisation with $hGPI_{544-558}$ could not even induce $hGPI_{544-558}$-specific Th17 cells.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

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

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

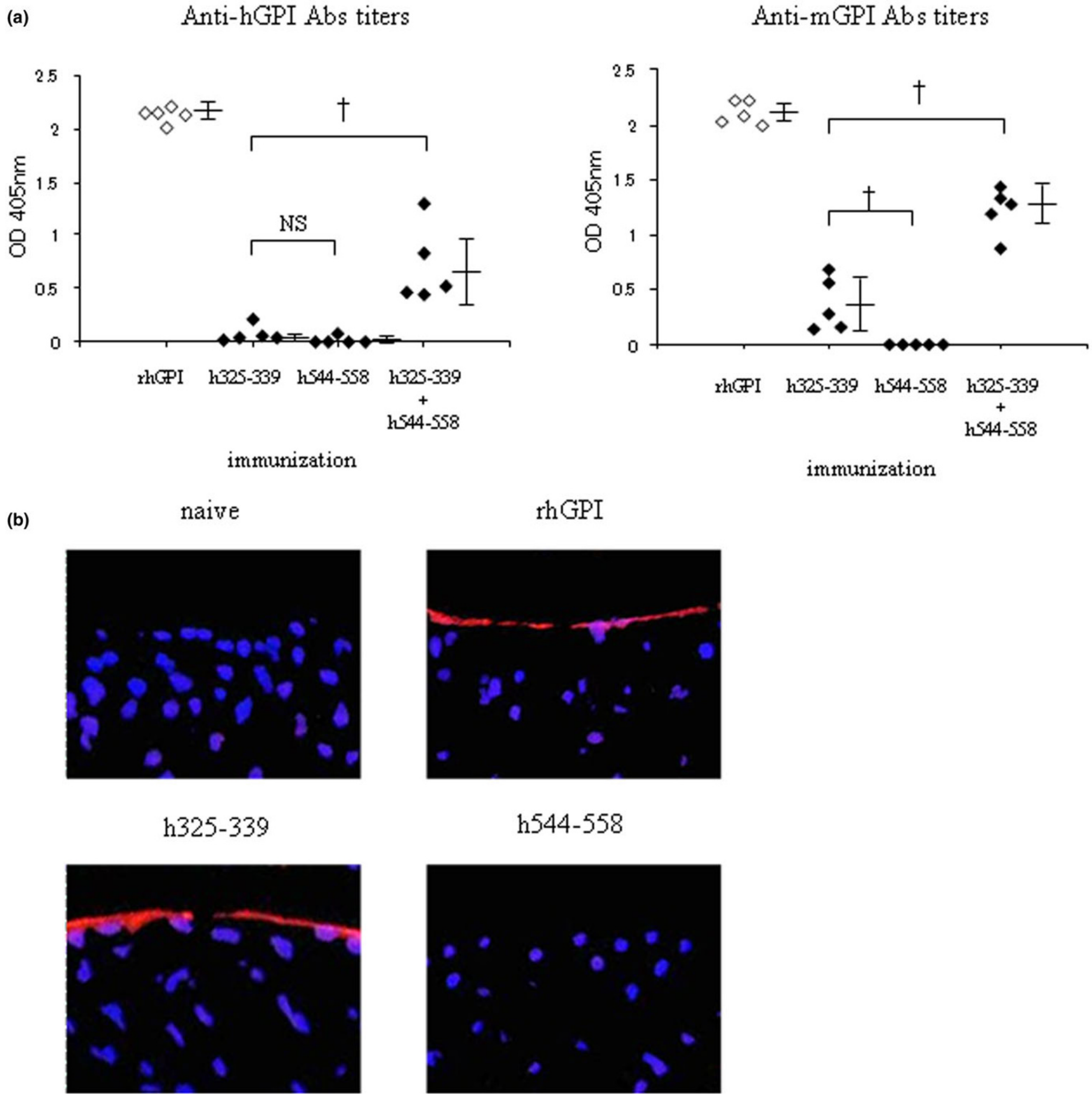
From a probability point of view, it is possible that other epitopes exist in some regions of human GPI-amino acid

Figure 5



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

Figure 6



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

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

Table 5

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

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

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

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

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

Conclusions

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

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

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

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