that M3R reactive immune reaction plays a crucial role in the pathogenesis of SS-like autoimmune sialoadenitis.

Take-home messages

- Anti-M3R Abs were detected in 50% of Sjögren's Syndrome (SS) patients, suggesting the possible serological marker for diagnosis of SS.
- The functional difference among anti-M3R antibodies against distinct B cell epitopes should shed light on the pathogenic roles of anti-M3R Abs in salivary secretion abnormalitites in SS patients.
- Murine model for autoimmune sialoadenitis using M3R$^{-/-}\rightarrow$ Rag-1$^{-/-}$ mice clearly showed that M3R reactive T cells play a crucial role in the generation of SS-like autoimmune sialoadenitis.

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Indirect inhibition of in-vivo and in-vitro T-cell responses by intravenous immunoglobulins due to impaired antigen presentation

Several clinical studies done with intravenous immunoglobulin (IVIg)-treated autoimmune patients as well as several in vitro studies have revealed that IVIg can reduce polyclonal T-cell activation and modify their cytokine secretion pattern. However, their effect on auto-antigen-specific T-cell responses has never been addressed directly. In the present study, **Aubin E. et al. (Blood 2010; 115: 1727-34)** used an in vivo model of induction of antigen-specific T-cell responses and an in vitro antigen presentation system to study the effects of IVIg on T-cell responses. The results obtained showed that IVIg inhibited both the in vivo and in vitro antigen-specific T-cell responses but that this effect was the indirect consequence of a reduction in the antigen presentation ability of antigen=presenting cells. The inhibitory effect of IVIg was FcgammaRIIb-independent, suggesting that IVIg must interfere with activating FcgammaRs expressed on antigen-presenting cells to reduce their ability to present antigens. Such inhibition of T-cell responses by reducing antigen presentation may therefore contribute to the well-known anti-inflammatory effects of IVIg in autoimmune diseases.

Rituximab treatment overcomes reduction of regulatory iNKT cells in patients with rheumatoid arthritis

Invariant natural killer T (iNKT) cells are subset of T cells that recognize glycolipid antigens presented by the CD1d molecule. Accumulating evidences showed that iNKT cells are implicated in the regulatory mechanisms that control autoimmunity. Here, Parietti V. et al. (Clin Immunol 2010; 134: 331-9) evaluated the number of circulating iNKT cells in patients with rheumatoid arthritis (RA) by flow cytometry and performed a longitudinal analysis of iNKT cell frequency in RA patients who were given an anti-CD20 therapy. Significantly lower iNKT cell numbers were measured in the blood from RA patients compared to healthy individuals ($p < 0.0001$) and low iNKT cell frequencies were rather associated with an active disease. In RA patients who received rituximab treatment, iNKT cell number was increased in relation to the clinical outcome. Thus, it was demonstrated that the number of iNKT cells is altered in RA patients and that following rituximab therapy, clinical remission of RA is associated with an increase of iNKT cell frequency.

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Research article

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Altered peptide ligands inhibit arthritis induced by glucose-6-phosphate isomerase peptide

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Abstract

Introduction Immunosuppressants, including anti-TNFα antibodies, have remarkable effects in rheumatoid arthritis; however, they increase infectious events. The present study was designed to examine the effects and immunological change of action of altered peptide ligands (APLs) on glucose-6-phosphate isomerase (GPI) peptide-induced arthritis.

Methods DBA/1 mice were immunized with $hGPI_{325-339}$, and cells of draining lymph node (DLN) were stimulated with $hGPI_{325-339}$ to investigate the T-cell receptor (TCR) repertoire of antigen-specific $CD4^+$ T cells by flow cytometry. Twenty types of APLs with one amino acid substitution at a TCR contact site of $hGPI_{325-339}$ were synthesized. $CD4^+$ T cells primed with human GPI and antigen-presenting cells were co-cultured with each APL and cytokine production was measured by ELISA to identify antagonistic APLs. Antagonistic APLs were co-immunized with $hGPI_{325-339}$ to investigate whether arthritis could be antigen-specifically inhibited by APL. After co-immunization, DLN cells were stimulated with $hGPI_{325-339}$ or APL to investigate Th17 and regulatory T-cell population by flow cytometry, and anti-mouse GPI antibodies were measured by ELISA.

Results Human $GPI_{325-339}$-specific Th17 cells showed predominant usage of TCRVβ8.1 8.2. Among the 20 synthesized APLs, four (APL 6; N329S, APL 7; N329T, APL 12; G332A, APL 13; G332V) significantly reduced IL-17 production by $CD4^+$ T cells in the presence of $hGPI_{325-339}$. Co-immunization with each antagonistic APL markedly prevented the development of arthritis, especially APL 13 (G332V). Although co-immunization with APL did not affect the population of Th17 and regulatory T cells, the titers of anti-mouse GPI antibodies in mice co-immunized with APL were significantly lower than in those without APL.

Conclusions We prepared antagonistic APLs that antigen-specifically inhibited the development of experimental arthritis. Understanding the inhibitory mechanisms of APLs may pave the way for the development of novel therapies for arthritis induced by autoimmune responses to ubiquitous antigens.

Introduction

Rheumatoid arthritis (RA) is characterized by symmetrical polyarthritis and joint destruction. Although the etiology is considered autoimmune reactivity to some antigens, the exact mechanisms are not fully understood. Pathological examinations show that most of the lymphocytes infiltrating the synovium in RA are $CD4^+$ T cells, which can recognize some antigens and expand oligoclonally intraarticularly [1]. These findings imply the possible role of $CD4^+$ T cells in the pathogenesis of RA. Previous studies showed that cytotoxic T-lymphocyte antigen-4 immunoglobulin and tacrolimus have remarkable effects on RA, and stressed the importance of $CD4^+$ T cells in the pathogenesis of RA [2-4].

Ab: antibody; APC: antigen-presenting cell; APL: altered peptide ligand; CII: collagen type II; DLN: draining lymph node; ELISA: enzyme-linked immunosorbent assay; GPI: glucose-6-phosphate isomerase; IFN: interferon; IL: interleukin; MBP: myelin basic protein; MHC: major histocompatibility complex; PBS: phosphate-buffered saline; PLP: proteolipid protein; RA: rheumatoid arthritis; rhGPI: recombinant human glucose-6-phosphate isomerase; TCR: T-cell receptor; Th: T-helper; TNF: tumor necrosis factor.

Although the exact helper T-cell lineage critical in RA remains elusive, previous animal studies reported that Th17 cells play a crucial role and that Th1 cells may have a protective role against the progress of arthritis in most mouse models with the exception of proteoglycan-induced arthritis in Balb/c mice [5]. Collagen-induced arthritis in the C57BL/6 background is markedly suppressed in IL-17-deficient mice [6], and glucose-6-phosphate isomerase (GPI)-induced arthritis in the DBA/1 background and antigen-induced arthritis in the C57BL/6 background are also suppressed by the administration of anti-IL-17 antibodies (Abs) [7,8]. In these models, complete Freund's adjuvant is used for the induction of arthritis; therefore it is possible that the components of *Mycobacterium tuberculosis* affect the cytokine dependency. The arthritis seen in IL-1 receptor antagonist-deficient mice in the Balb/c background and SKG mice in the Balb/c background, however, is completely suppressed in IL-17-deficient mice [9,10]. These findings indicate that Th17 cells play a central role in murine models independent of mouse strains and target antigens.

IL-17 is also considered to play a crucial role in host defense. IL-17 signaling seems essential for the recruitment of neutrophils to the alveolar space in pneumonia caused by *Klebsiella pneumoniae*, *Mycoplasma pneumoniae* and *Pneumocystis jiroveci* [11-13]. IL-17 is also involved in mucosal host defense against oropharyngeal candidasis via salivary antimicrobial factors, in addition to neutrophil recruitment [14]. Furthermore, IL-17 production by γδ T cells is essential against peritonitis caused by *Escherichia coli* [15]. In this regard, anti-cytokine therapies such as infliximab and tocilizumab have been applied to clinical treatment and have shown striking effects on RA [16-19]; anti-IL-17 therapy could therefore be useful in the treatment of RA. Blockade of IL-17 could increase the likelihood of infections, however, and the use of such a strategy would be limited just like the case of infliximab and tocilizumab.

Altered peptide ligands (APLs) are peptides with substitutions in amino acid residues at T-cell receptor (TCR) contact sites, and can be either agonistic, antagonistic with partial activation or antagonistic [20]. These three different actions seem to depend on the site and residue of the peptide substitution [21]. The antagonistic APLs can inhibit the function of limited T-cell populations, and thus they could be potentially useful as antigen-specific therapy for autoimmune diseases in which T cells play a pathogenic role. Indeed, APLs have been proven effective in the suppression of several autoimmune models. In an arthritis model, previous studies identified type II collagen $CII_{245-270}$ as a prominent T-cell epitope in collagen-induced arthritis in DBA/1 mice, and found that co-immunization with the analog peptide (I260A, A261B(hydroxyproline), F263N), also known as A9, significantly suppressed the disease [22,23]. As reported previously, however, the type II collagen residues CII 260 (I) and CII 263 (F) are I-Aq (MHC class II of DBA/1 mice) binding sites, and A9 was confirmed not to bind I-Aq molecules [24,25]. The analog peptide A9 therefore seems to differ from conventional APLs, and the inhibitory effect and the mechanisms of conventional APLs on arthritis remain to be defined.

Several models of arthritis have so far been described and analyzed to understand the etiological mechanisms of RA. GPI-induced arthritis, a murine model of RA, is induced by immunization of DBA/1 mice with recombinant human GPI (rhGPI) [26]. We demonstrated previously that the Th17 subset of $CD4^+$ T cells played a central role in the pathogenesis of GPI-induced arthritis; GPI-specific $CD4^+$ T cells were skewed to T_H-17 at the time of onset, and blockade of IL-17 resulted in a significant amelioration of arthritis [7]. We have also demonstrated that the major epitope of $CD4^+$ T cells in GPI-induced arthritis was $hGPI_{325-339}$, and immunization with the peptide induced severe polyarthritis (GPI peptide-induced arthritis) [27].

The present work is an extension to the above studies. Specifically, we explored the antigen-specific Th17 inhibition, explored the effects of APLs on arthritis, and investigated the inhibitory mechanisms of APLs, using the T-cell-dependent model of GPI peptide-induced arthritis. The results showed that many $hGPI_{325-339}$-specific $CD4^+$ T cells employed Vβ8.1 8.2 as the TCR repertoire, and co-immunization with APL (N329S, N329T, G332A, or G332V) significantly inhibited the development of arthritis. Our analysis of the inhibitory mechanisms of APLs indicates that our APLs can function as TCR antagonists; however, they can differentiate naïve $CD4^+$ T cells to Th17 cells, but not Th2 cells or regulatory T cells. Based on these findings, we define a new aspect for APLs, and propose that they may provide the basis for the invention of new antigen-specific therapy.

Materials and methods

Mice

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

Glucose-6-phosphate isomerase and synthetic peptides

The rhGPI and recombinant mouse GPI were prepared as described previously [28,29]. Briefly, human or mouse GPI cDNA was inserted into the plasmid pGEX-4T3 (Pharmacia, Uppsala, Sweden) for expression of glutathione-*S*-transferase-tagged proteins. *E. coli* harboring pGEX-hGPI plasmid was allowed to proliferate at 37°C, before the addition of 0.1 mM isopropyl-β-d-thiogalactopyranoside 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). The purity was estimated by SDS-PAGE.

Peptides for screening were synthesized with 70% purity by Wako Pure Chemical Industries, Ltd (Osaka, Japan), and peptides of a major peptide and antagonistic altered peptide ligands were synthesized with 90% purity by Invitrogen (Carlsbad, CA, USA). $OVA_{323-339}$ peptide was purchased from AnaSpec (San Jose, CA, USA).

Induction of arthritis

DBA/1 mice were immunized with 10 μg synthetic peptides for GPI peptide-induced arthritis in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA), and in the indicated experiments 50 μg altered peptide ligands were used with 10 μg $GPI_{325-339}$ for co-immunization. The synthetic peptides were emulsified with complete Freund's adjuvant at a 1:1 ratio (v/v). For induction of arthritis, 150 μl emulsion was injected intradermally at the base of the tail, and 200 ng pertussis toxin was injected intraperitoneally on days 0 and 2 after immunization.

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 localized edema, 2 represented easily identified swelling that was localized to either the dorsal or ventral surface of the paws, and 3 represented swelling in all areas of the paws.

Screening of antagonistic altered peptide ligands

Mice were sacrificed on the indicated day. Spleens and draining lymph nodes (DLNs) were harvested, and splenocytes were hemolyzed 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 RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. $CD4^+$ T cells from DLNs and $CD11c^+$ dendritic cells from spleens were isolated by magenetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the collected cells (>97%) was confirmed by flow cytometry. $CD11c^+$ dendritic cells treated with 50 μg/ml mitomycin C were used as antigen-presenting cells (APCs). The purified $CD4^+$ T cells and APCs were co-cultured with 10 μM synthetic peptide at a ratio of 1:3 in 96-well round-bottom plates (Nunc, Roskilde, Denmark) at 37°C under 5% carbon dioxide for 72 hours. The supernatants were assayed for IL-10 and IL-17 by the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

Pre-pulse assay

The pre-pulse assay was conducted as described previously [30]. Briefly, $CD11c^+$ APCs from spleens (4×10^4/well) were cultured with a suboptimal concentration of $GPI_{325-339}$ (3 μM) for 2 hours. In the meantime, native peptides were loaded onto APCs and presented on MHC. After 2 hours of incubation, APCs were washed twice to remove unbound peptides, and 30 μM each antagonistic APL was added. After 18 hours of culture, $CD4^+$ T cells (2×10^4/well) from DLNs were added, and after an additional 72 hours of culture the supernatants were assayed for IL-17 and IL-10 by ELISA. The inhibition ratio was calculated as follows:

1 – (IL-17 concentration in the presence of native peptides and APLs / IL-17 concentration in the presence of native peptides only) x 100 (%)

Flow cytometry

Mice were sacrificed on the indicated day. The popliteal lymph nodes were harvested and single-cell suspensions were prepared as described above. Cells (1×10^6/ml) were stimulated with 100 μg/ml rhGPI in 96-well round-bottom plates (Nunc) for 24 hours and GoldiStop (BD PharMingen, San Diego, CA, USA) was added for the last 2 hours of each culture. Cells were first stained extracellularly, fixed and permeabilized with Cytofix/Cytoperm solution (BD PharMingen) and then stained intracellularly. Regulatory T cells were stained with the Mouse Regulatory T cell Staining kit (eBioscience, San Diego, CA, USA) according to the protocol supplied by the manufacturer. For TCR repertoire screening, the Mouse TCR Screening Panel (BD PharMingen) was used. Samples were acquired on FACSCalibur (BD PharMingen) and data were analyzed with FlowJo (Tree Star, Ashland, OR, USA).

Analysis of anti-glucose-6-phosphate isomerase antibody

Sera were taken from immunized mice on day 28 and were diluted 1:500 (for IgG, IgG_{2a}, IgG_{2b} and IgG_3) or 1:8,000 (for IgG_1) 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 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 2 hours at room temperature. After two washes, 150 μl diluted serum was added and incubated for 2 hours at room temperature. After three washes, alkaline phosphatase-conjugated anti-mouse IgG, horseradish peroxidase-conjugated anti-mouse IgG_1, IgG_{2a}, IgG_{2b} (Zymed Laboratories, San Francisco, CA, USA) or IgG_3 (Invitrogen) was added at a final dilution of 1:5,000 for 1 hour at room temperature. After three washes, color was developed with substrate solution (1 alkaline phosphatase tablet (Sigma-Aldrich) per 5 ml alkaline phosphatase reaction solution (containing 9.6% diethanolamine and 0.25 mM $MgCl_2$, pH 9.8)) or tetramethylbenzidine (KPL, Gaithersburg, MD, USA). Plates were incubated for 20 to 60 minutes at room temperature and the optical density was measured by a microplate reader at 405 nm (for IgG) or at 450 nm (for IgG_1, IgG_{2a}, IgG_{2b} and IgG_3).

Statistical analysis

All data are expressed as the mean ± standard error of the mean or standard deviation. Differences between groups were

examined for statistical significance using the Mann-Whitney U test. Differences of arthritis incidence between groups were examined using Fisher's exact test. $P < 0.05$ denotes the presence of a statistically significant difference.

Results

Designing and screening antagonistic altered peptide ligands

We reported previously that the major T-cell epitope in GPI-induced arthritis is $hGPI_{325-339}$, and immunization with the peptide provokes symmetrical polyarthritis (GPI peptide-induced arthritis) [28]. To investigate the effects of APLs in GPI peptide-induced arthritis, we first designed APLs of $hGPI_{325-339}$. Since the MHC binding sites of $hGPI_{325-339}$ exist at P1 (I328), P4 (F331), and P7 (E334) (IWYINCEGCETHAML) [25,28], the amino acid residues of the TCR contact sites at P0 (Y327), P2 (N329), P3 (C330), P5 (G332), P6 (C333), and P8 (T335) were substituted for another peptide to design 20 types of APLs (Table 1).

To select antagonistic APLs, CD4+ T cells primed with rhGPI and APCs were co-cultured with each APL. The IL-17 production was markedly lower with APL 2, APL 5, APL 6, APL 7, APL 9, APL 10, APL 11, APL 12, APL 13, and APL 18 than with $hGPI_{325-339}$, and therefore these APLs were considered candidates of antagonistic APLs (Figure 1a). None of the APLs induced IL-4 and IL-10 production (data not shown). We next explored the potency of the APLs in inhibiting IL-17 production in the presence of $hGPI_{325-339}$. In the pre-pulse assay, APL 6 (N329S), APL 7 (N329T), APL 12 (G332A), and APL 13 (G332V) significantly reduced IL-17 production by CD4+ T cells primed with rhGPI in the presence of $hGPI_{325-339}$ ($P < 0.01$) (Figure 1b). We therefore considered these four APLs as antagonistic APLs.

Inhibition of arthritis by antagonistic altered peptideligands

Since GPI peptide-induced arthritis is mediated by Th17 and antagonistic APLs can suppress IL-17 production, we explored the efficacy of the prepared APLs on the inhibition of

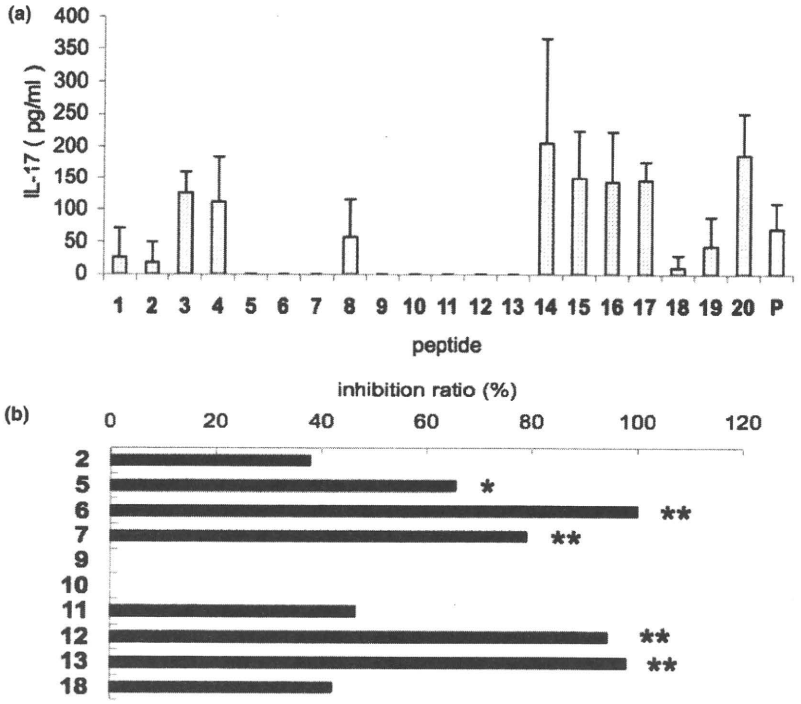
Table 1

$hGPI_{325-339}$-derived altered peptide ligands used in the present study

325 to 339	I	W	Y	I	N	C	E	G	C	E	T	H	A	M	L
APL 1	--	--	N		--	--	--	--	--	--	--	--	--	--	--
APL 2	--	--	Q		--	--	--	--	--	--	--	--	--	--	--
APL 3	--	--	S		--	--	--	--	--	--	--	--	--	--	--
APL 4	--	--	T		--	--	--	--	--	--	--	--	--	--	--
APL 5	--	--	--		Q	--		--	--		--	--	--	--	--
APL 6	--	--	--		S	--		--	--		--	--	--	--	--
APL 7	--	--	--		T	--		--	--		--	--	--	--	--
APL 8	--	--	--		--	N		--	--		--	--	--	--	--
APL 9	--	--	--		--	Q		--	--		--	--	--	--	--
APL 10	--	--	--		--	S		--	--		--	--	--	--	--
APL 11	--	--	--		--	T		--	--		--	--	--	--	--
APL 12	--	--	--		--	--		A	--		--	--	--	--	--
APL 13	--	--	--		--	--		V	--		--	--	--	--	--
APL 14	--	--	--		--	--		--	N		--	--	--	--	--
APL 15	--	--	--		--	--		--	Q		--	--	--	--	--
APL 16	--	--	--		--	--		--	S		--	--	--	--	--
APL 17	--	--	--		--	--		--	T		--	--	--	--	--
APL 18	--	--	--		--	--		--	--		N	--	--	--	--
APL 19	--	--	--		--	--		--	--		Q	--	--	--	--
APL 20	--	--	--		--	--		--	--		S	--	--	--	--

The MHC binding sites exist at glucose-6-phosphate isomerase (GPI) 328 (I), GPI 331 (F) and GPI 334 (E) as indicated (underlined). The amino acid residues of the T-cell receptor contact sites at P0 (Y327), P2 (N329), P3 (C330), P5 (G332), P6 (C333), and P8 (T335) were substituted for the indicated peptides to design 20 types of altered peptide ligands (APLs).

Figure 1



Altered peptide ligands markedly suppress IL-17 production by glucose-6-phosphate isomerase-primed CD4+ T cells. Altered peptide ligand (APL) 6, APL 7, APL 9, APL 12 and APL 13 markedly suppress IL-17 production by glucose-6-phosphate isomerase (GPI)-primed CD4+ T cells. Mice were sacrificed on day 8 after immunization. CD4+ T cells were purified from draining lymph node cells of recombinant human GPI (hGPI)-immunized DBA/1 mice, and CD11c+ antigen-presenting cells (APCs) were purified from spleen cells. **(a)** CD4+ T cells primed with hGPI and APCs were co-cultured with 10 μM synthetic peptide for 72 hours. The supernatants were assayed for IL-17 by ELISA. P, positive control ($hGPI_{325-339}$). **(b)** CD11c+ APCs were cultured with a suboptimal concentration $GPI_{325-339}$ (3 μM) for 2 hours, washed twice to remove unbound peptides, and 30 μM each antagonistic APL was added. After 18 hours of culture, CD4+ T cells (2×10^4/well) were added, and after an additional 72 hours of culture, the supernatants were assayed for IL-17 by ELISA. The inhibition ratio was calculated as stated in Pre-pulse assay. Data presented as average ± standard deviation of three culture wells. $^*P < 0.05$, $^{**}P < 0.01$ (Mann--Whitney U test). Representative data of two independent experiments.

arthritis. First, we immunized DBA/1 mice with each APL alone, and confirmed that no overt arthritis developed (data not shown). DBA/1 mice were then co-immunized with $hGPI_{325-339}$ and each APL to explore the development of arthritis. Mice co-immunized with APL 6, APL 12 and APL 13 developed significantly attenuated arthritis after day 12, and those co-immunized with APL 7 after day 16, compared with mice immunized with $hGPI_{325-339}$ alone ($P < 0.05$) (Figure 2, upper panel). Co-immunization with APL 13 also significantly suppressed the incidence of arthritis ($P < 0.05$) (Table 2). Co-immunization with $hGPI_{325-339}$ and APL 15, an agonistic APL, however, did not affect the severity or course of arthritis (Figure 2, middle panel). Moreover, mice co-immunized with $hGPI_{325-339}$ and $OVA_{323-339}$ also had a similar course of arthritis to $hGPI_{325-339}$ alone (Figure 2, lower panel).

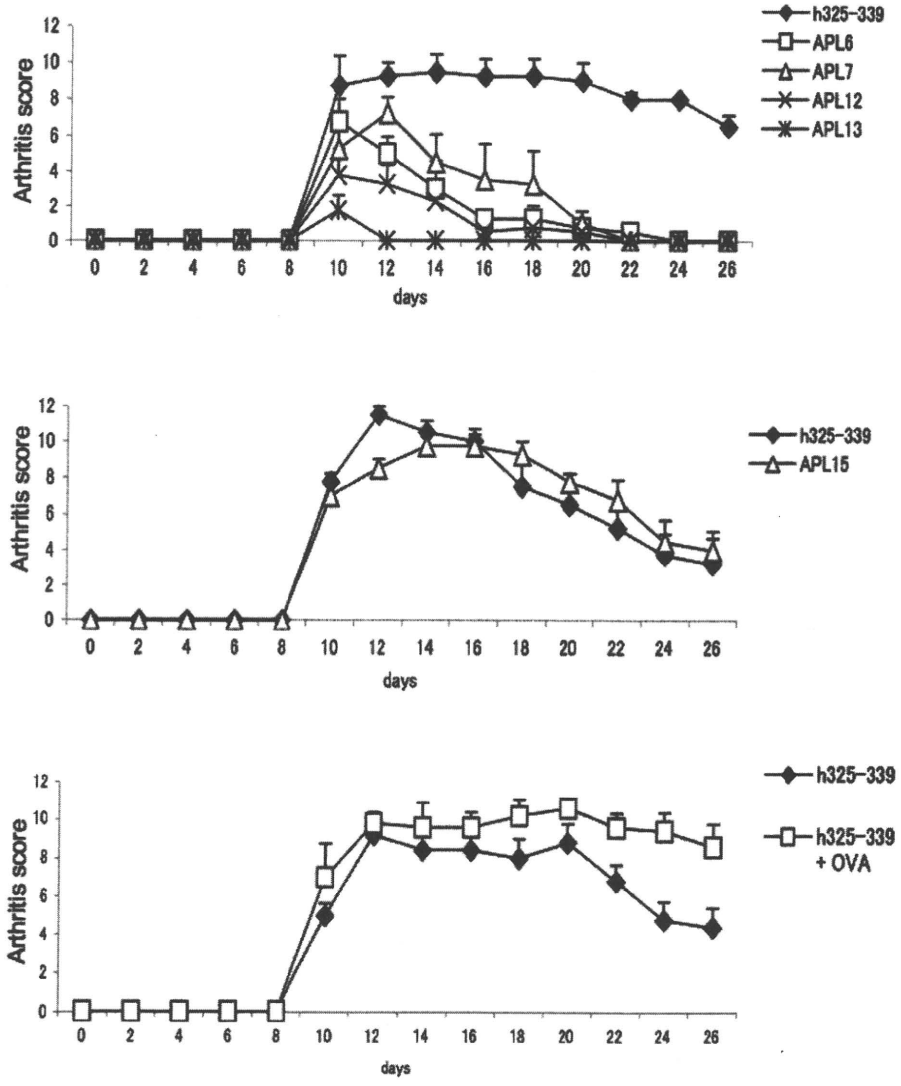
Identification of TCRVβ usage of $hGPI_{325-339}$-specific Th17 cells

To investigate the inhibitory mechanisms of the antagonistic APLs, we explored TCRVβ usage of $hGPI_{325-339}$-specific CD4+ T cells. The CD4+ T cells primed with $hGPI_{325-339}$ were stimulated with $hGPI_{325-339}$ *in vitro* and the TCRVβ repertoire was analyzed by flow cytometry and compared with that before stimulation. Stimulation with $hGPI_{325-339}$ expanded the population of CD4+ T cells with TCRVβ8.1 8.2 (Figure 3a). We also found that much of IL-17 was produced by CD4+ T cells with TCRVβ8.1 8.2 following stimulation with $hGPI_{325-339}$ (Figure 3b). These data indicate that many $hGPI_{325-339}$-specific Th17 cells use TCRVβ8.1 8.2.

Effect of antagonistic altered peptide ligands on differentiation of Th17 and regulatory T cells

In vitro analysis showed that the antagonistic APLs suppressed IL-17 production, and that co-immunization with the APLs inhibited the development of arthritis. We therefore explored the effect of APLs on Th17 differentiation. Our previous report suggested that cross-reactivity of CD4+ T cells primed with $hGPI_{325-339}$ to $mGPI_{325-339}$ was critical for the development of arthritis. We therefore assessed the population of $mGPI_{325-339}$ reactive Th17 cells in the DLNs of mice co-

Figure 2



Co-immunization with antagonistic altered peptide ligands suppresses the development of arthritis. Mice were co-immunized with antagonistic altered peptide ligand (APL) 6, APL 7, APL 12, APL 13 (upper panel), the agonistic APL 15 (middle panel) or OVA peptide (lower panel). Progression of arthritis was significantly suppressed in mice co-immunized with APL 6, APL 12 and APL 13 after day 12, and in mice co-immunized with APL 7 after day 16 ($P < 0.05$, Mann--Whitney U test). Data presented as mean arthritis score (± standard error of the mean) of four mice in one representative experiment of at least two independent experiments.

immunized with each APL. IL-17 production by CD4+ T cells with TCRVβ8.1 8.2 or other TCRVβ with stimulation of $mGPI_{325-339}$was not affected by co-immunization with APLs (Figure 4a), and neither was affected IL-17 production with $hGPI_{325-339}$ (data not shown). Unexpectedly, IL-17 production was considerable with stimulation of the corresponding APLs (Figure 4b). ELISA showed undetectable levels of IL-4, and the IL-10 production, and IFNγ production was not affected (data not shown). Co-immunization with APLs did neither affect the population of regulatory T cells nor the expression of CD25 (Figure 5), and stimulation of DLN cells of co-immunized mice with APLs *in vitro* did not induce the expansion of regulatory T cells (data not shown).

Identification of TCRVβ usage of altered peptide ligand-specific CD4+ T cells

The unexpected data mentioned above suggested that APL-specific CD4+ T cells were developed by co-immunization. We

Table 2

Effects of co-immunization with altered peptide ligands on the development of arthritis

Co-immunization	Incidence	Day of onset	Maximum severity
None	8/8	10 ± 0.0	10.9 ± 1.4
APL 6	8/8	10 ± 0.0	7.5 ± 2.0*
APL 7	6/8	10 ± 0.0	7.8 ± 1.7*
APL 12	7/8	10.3 ± 0.8	5.0 ± 1.2**
APL 13	3/8†	10 ± 0.0	4.0 ± 0.0**

Mice were co-immunized with 10 μg glucose-6-phosphate isomerase $hGPI_{325-339}$and 50 μg indicated antigen. Data presented as incidence or mean ± standard deviation. †$P < 0.05$ (Fisher's exact test). *$P < 0.005$, **$P < 0.001$ (Mann--Whitney U test).

therefore investigated TCRVβ usage of APL-specific $CD4^+$ T cells. The $CD4^+$ T cells primed with each APL were stimulated with the corresponding APL *in vitro* and the TCRVβ repertoire was analyzed by flow cytometry and compared with that before stimulation. Stimulation with APL 6, APL 7 and APL 12 induced expansion of the population of $CD4^+$ T cells with TCRVβ8.1 8.2; however, this expansion was not so remarkable as that of $hGPI_{325-339}$-specific $CD4^+$ T cells (Figures 3a and 6). Interestingly, stimulation with APL 13 hardly induced the expansion of the population of $CD4^+$ T cells with TCRVβ8.1 8.2 (Figure 6) or any other specific Vβ chain, although each APL stimulation could proliferate $CD4^+$ T cells primed with the corresponding APL as efficiently as $hGPI_{325-339}$ (data not shown).

Effects of antagonistic altered peptide ligands on anti-mouse glucose-6-phosphate isomerase antibody production

Since administration of anti-CD4 monoclonal Abs with immunization prevents the development of arthritis and completely inhibits the production of anti-mGPI Abs in GPI-induced arthritis, mGPI is considered a thymus-dependent antigen to the humoral immune response [26]. We therefore next investigated the effects of APLs on antibody production. Co-immunization with APL 6, APL 7, APL 12 and APL 13 significantly suppressed the titers of anti-mGPI Abs ($P < 0.01$, $P < 0.005$, $P < 0.001$ and $P < 0.001$, respectively) (Figure 7). We also investigated the anti-mGPI IgG isotype. Co-immunization with APL 7, APL 12 and APL 13 significantly suppressed the titer of anti-mGPI IgG_1 isotype ($P < 0.005$, $P < 0.001$ and $P < 0.01$, respectively). Any other anti-mGPI IgG isotype was hardly detected, however, and any bias to specific isotype was not found as an effect of APL.

Discussion

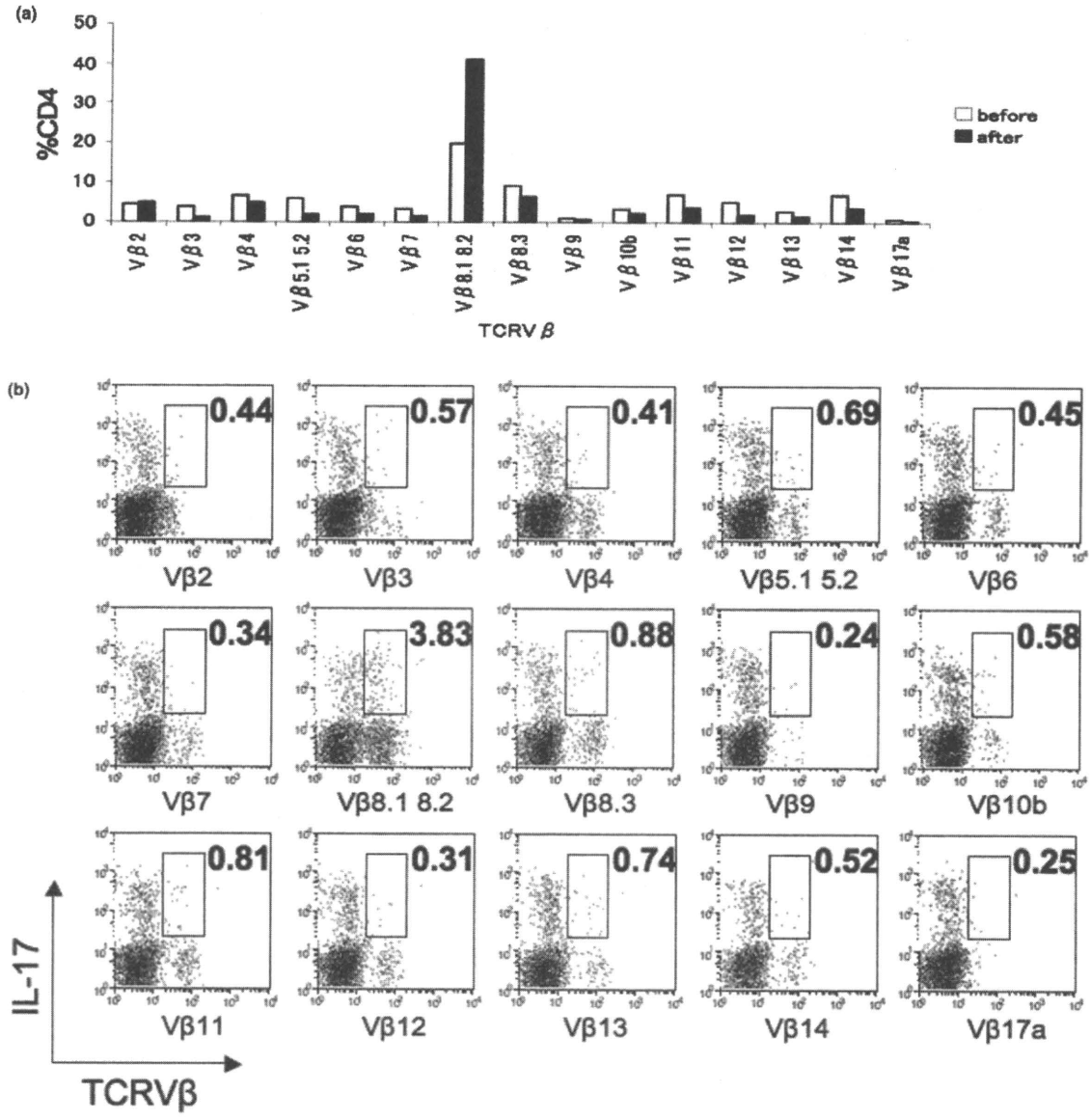
APLs are considered useful for antigen-specific therapy of autoimmune diseases and allergy. Treatments with APLs have so far been tested in several autoimmune models, and especially experimental autoimmune encephalitis has been enthusiastically examined for APLs designed as a single amino acid substitution of a TCR contact site. In experimental autoimmune encephalitis in Lewis rats, co-immunization with the APL (K91A) of myelin basic protein MBP_{87-99} strongly inhibited the development of the disease by suppression of IFNγ and TNFα, not T-cell proliferation [31]. Furthermore, another study of experimental autoimmune encephalitis in SJL mice showed that co-immunization with the APL (W144Q) of myelin proteolipid protein $PLP_{139-151}$ also inhibited the disease, and that the T-cell clone specific for the APL (W144Q) possessed the Th0 or Th2 phenotype [32].

Although one study used conventional APLs in collagen-induced arthritis [33], unconventional APLs with substitutions at MHC binding sites were mainly tested in arthritis models. Myers and colleagues reported that the analog peptide (I260A, A261B(hydroxyproline), F263N) significantly suppressed collagen-induced arthritis by inducing Th2 cells in DBA/1 mice [34]. They also reported the suppression of collagen-induced arthritis in HLA-DR1 and HLA-DR4 transgenic mice using other analog peptides with substitutions at MHC binding sites [35,36]. Another group reported also that the antigen-specific proinflammatory response to the human cartilage glycoprotein-39 (263 to 275) epitope was suppressed in DR4 transgenic mice by APLs with substitution at MHC binding sites [37].

In our study, we designed various APLs (N329S, N329T, G332A, or G332V) of $hGPI_{325-339}$ with substitutions at TCR contact sites, and showed that co-immunization with the individual APL significantly inhibited the development of arthritis. Although the APLs had antagonism to Th17 primed with hGPI cells *in vitro* (Figure 1), analysis of the mechanisms of the effect of co-immunizing APLs showed normal development of $hGPI_{325-339}$-specific Th17 cells and APL-specific Th17 cells *in vivo* (Figure 4). Co-immunization with $hGPI_{325-339}$ and the APL might have induced both $hGPI_{325-339}$-specific Th17 clones and APL-specific Th17 clones by the adjuvant effects of complete Freund's adjuvant and pertussis toxin.

Since both the TCR signal and the co-stimulatory signal are essential for priming of naïve T cells, our data suggested the potency of the APLs as agonists to some TCRs. It is likely that

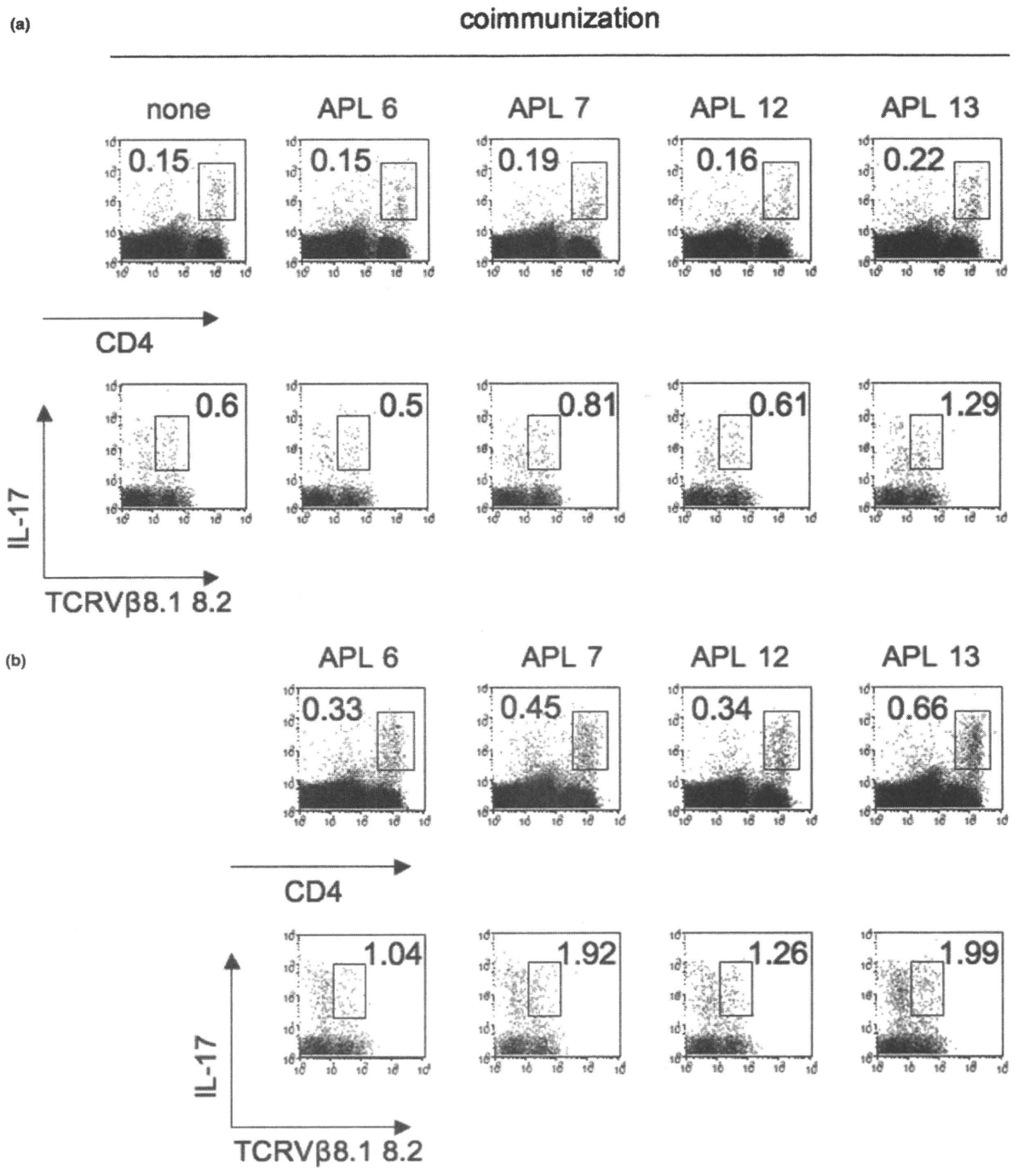
Figure 3



Human glucose-6-phosphate isomerase-specific Th17 cells use TCRVβ8.1 8.2. Many glucose-6-phosphate isomerase $hGPI_{325-339}$-specific Th17 cells use TCRVβ8.1 8.2. Mice were immunized with 10 μg $hGPI_{325-339}$, and draining lymph node cells on day 6 were stimulated with 20 μM $hGPI_{325-339}$ *in vitro*. **(a)** Ratios of TCRVβ repertoire to CD4+ T cells. The TCRVβ repertoire of CD4+ T cells was analyzed by flow cytometry: before stimulation with $hGPI_{325-339}$ *in vitro* for 72 hours, and after stimulation. **(b)** GoldiStop was added in the last 4 hours of the 24-hour culture. Flow cytometry analysis for IL-17 was gated in $CD4^{high}$ cells. Representative data of two independent experiments.

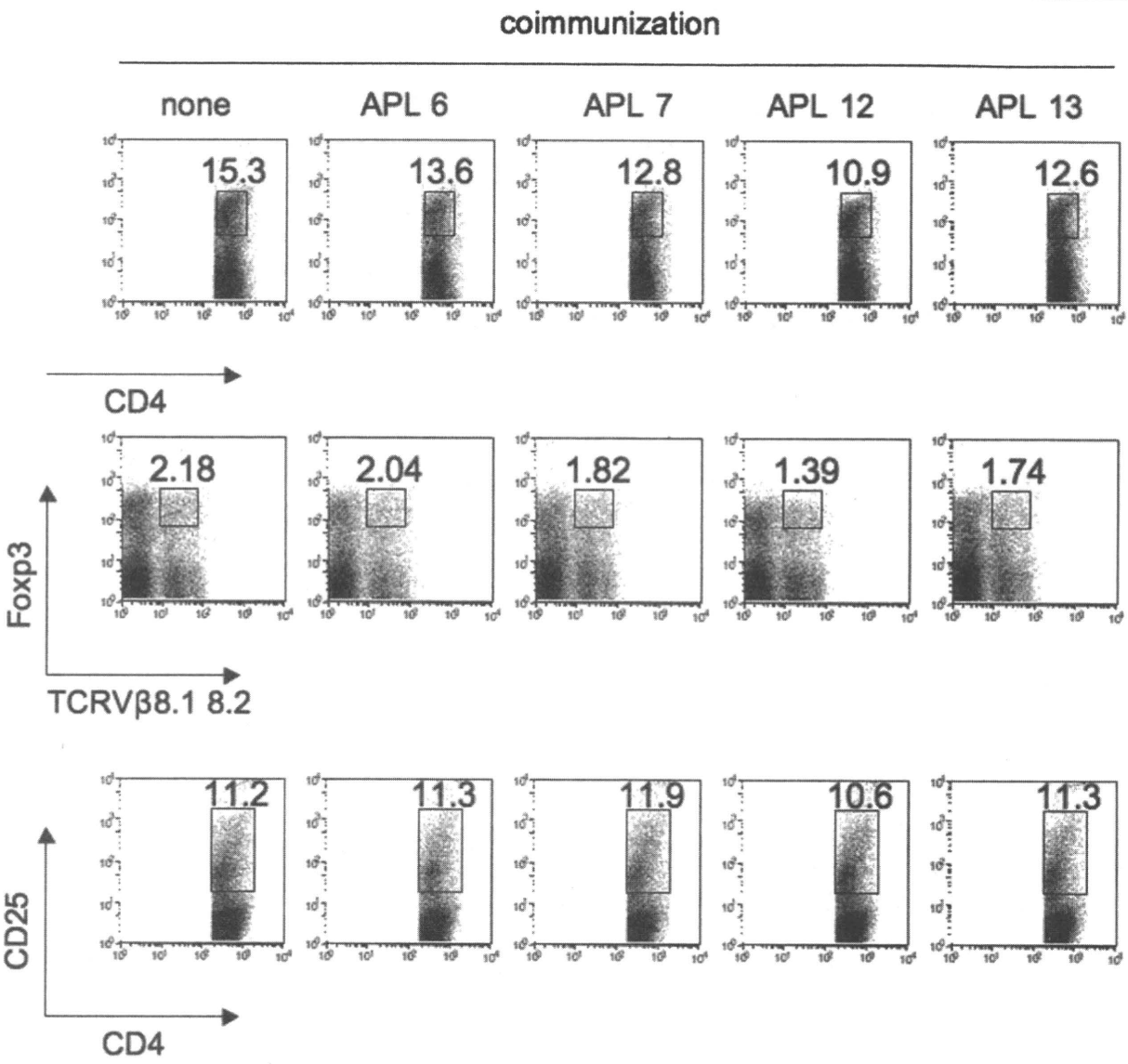
an antigen acts as an agonist to one T-cell clone and as an antagonist to another T-cell clone because any TCR can cross-react with various antigens. Although the antigen specificity is mainly determined by the complementary determining regions, the different ratio of TCRVβ usage between $hGPI_{325-339}$-specific CD4+ T cells and APL-specific CD4+ T cells (especially APL 13) indicates that each CD4+ T cell is a different clone that leads to different antigen specificity, and does not cross-react to the APLs and $hGPI_{325-339}$ to conduct positive TCR signals, respectively. Our previous paper showed that T cells primed with $hGPI_{325-339}$ could cross-react to $mGPI_{325-339}$ and that the cross-reactivity to $mGPI_{325-339}$ was crucial for induction arthritis [27]. The findings that immunization with the APLs (APL 6, APL 7, APL 12, APL 13) alone

Figure 4



Co-immunization with altered peptide ligands does not affect IL-17 production. Mice were immunized with 10 μg glucose-6-phosphate isomerase $hGPI_{325-339}$ and 50 μg each altered peptide ligand (APL). Draining lymph node cells on day 6 were stimulated for 24 hours *in vitro* **(a)** with 10 μM mouse $GPI_{325-339}$ or **(b)** with 10 μM corresponding APL. GoldiStop was added in the last 4 hours of each culture. Flow cytometry analysis for IL-17 and TCRVβ repertoire was gated in $CD4^{high}$ cells. None, immunization with no APL ($hGPI_{325-339}$alone). Representative flow cytometry data of two independent experiments.

Figure 5

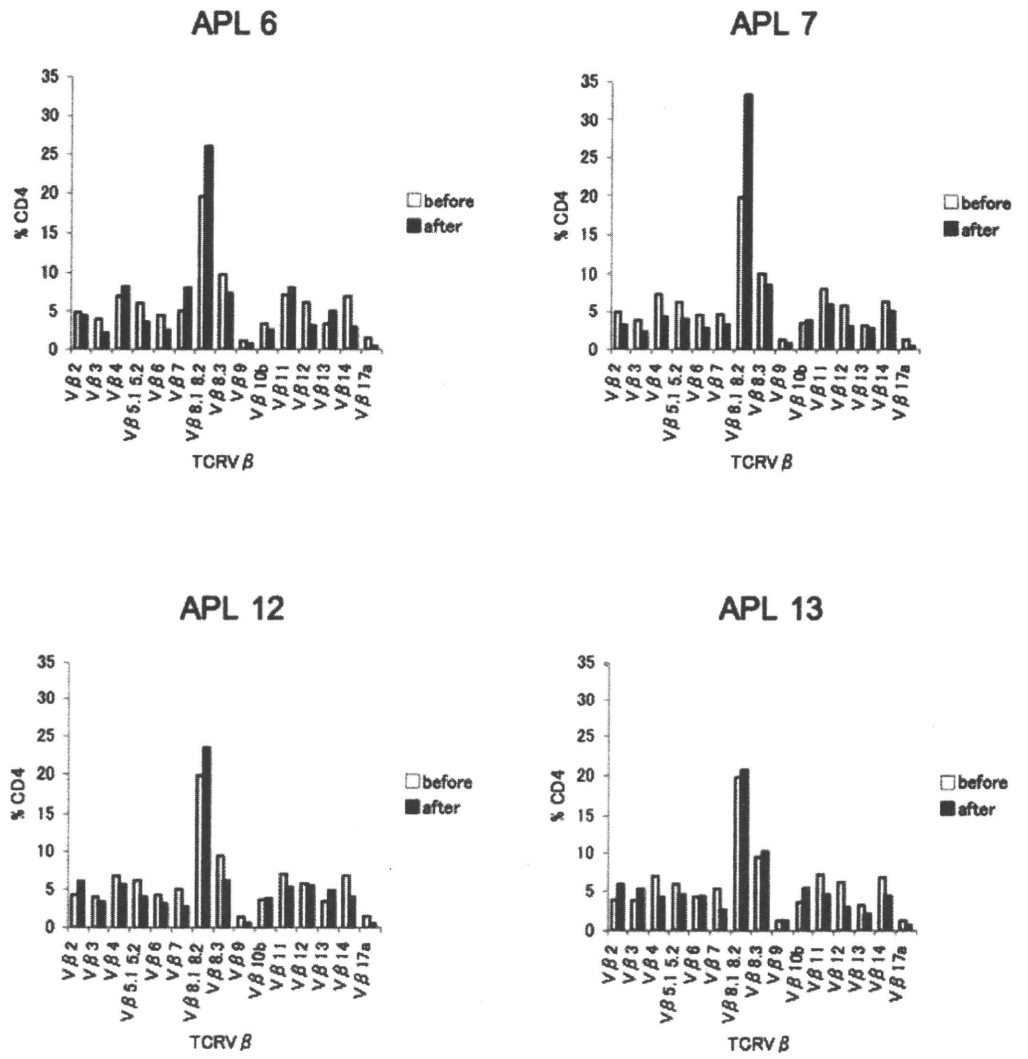


Co-immunization with altered peptide ligands neither induces regulatory T cells nor modulates CD25 expression. Mice were immunized with 10 μg glucose-6-phosphate isomerase $hGPI_{325-339}$ and 50 μg each altered peptide ligand (APL), and draining lymph node cells on day 6 were stained with Foxp3 and CD25. Flow cytometry analysis was gated in $CD4^+$ cells. None, immunization with no APL ($hGPI_{325-339}$ alone). Representative flow cytometry data of two independent experiments.

could not induce any overt arthritis indicated that APL-specific T cells could not cross-react $mGPI_{325-339}$ suggesting they do not have potential for induction of arthritis.

One of the inhibitory mechanisms of APL is anergy. Allen and colleagues reported that APL could induce anergy of T-cell clones by partial activation [38], which was characterized by an increase in cell volume and upregulation of CD25, without cytokine production or cell proliferation. Another mechanism is induction of anti-inflammatory T-cell lineages such as Th2/Th0 as well as regulatory T cells. Nicholson and colleagues reported that co-immunization with $PLP_{139-151}$ and APL (W144L/H147R) did not inhibit the induction of $PLP_{139-151}$-specific T cells, but induced APL-specific Th2/Th0 phenotype cells to suppress the progression of experimental autoimmune encephalitis by stander suppression [39].

Figure 6

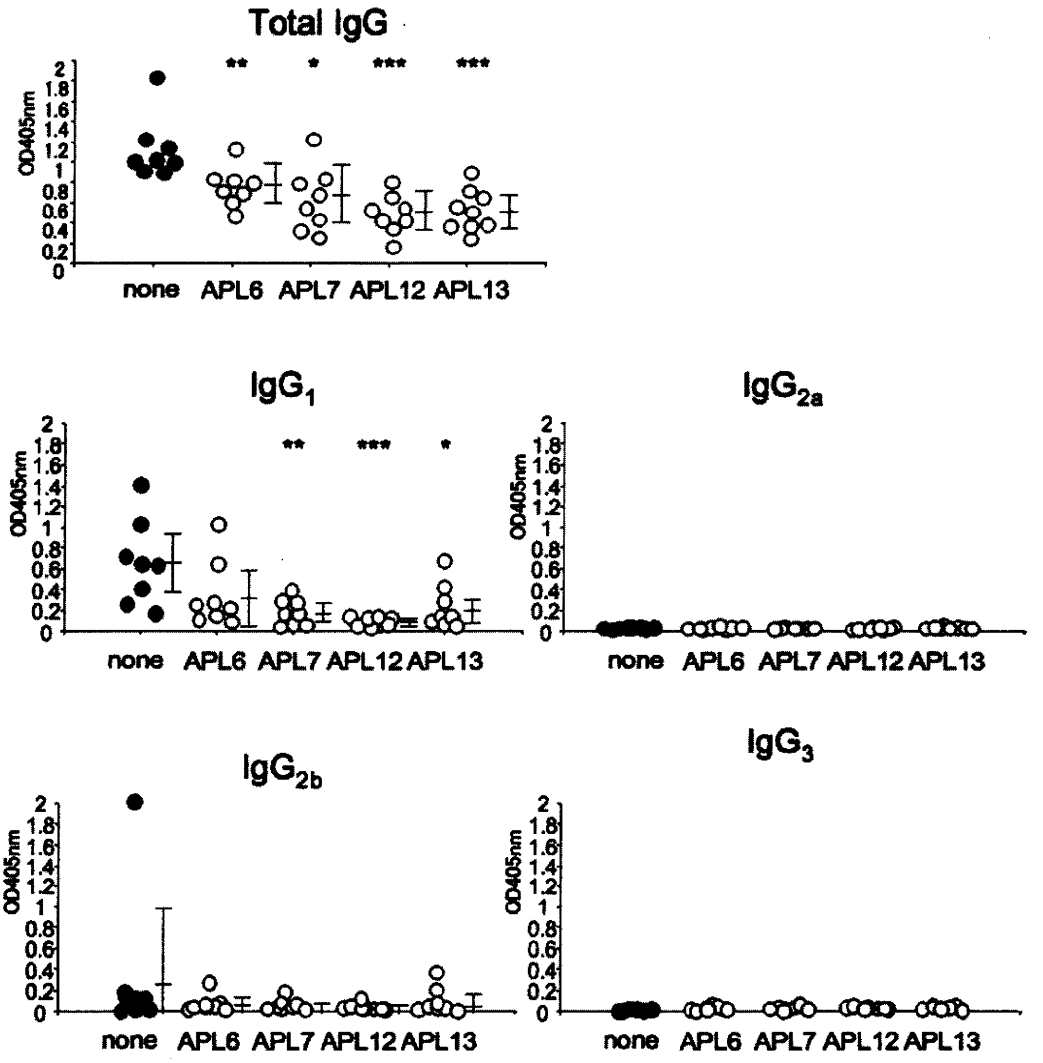


TCRVβ usage of altered peptide ligand-specific CD4+ T cells. TCRVβ usage of altered peptide ligand (APL)-specific CD4+ T cells was not remarkably shifted to TCRVβ8.1 8.2. Mice were immunized with 10 μg each APL, and draining lymph node cells on days 7 to 9 were stimulated with 20 μM corresponding APL *in vitro*. Ratios of TCRVβ repertoire to CD4+ T cells. The TCRVβ repertoire of CD4+ T cells was analyzed by flow cytometry: before stimulation with the corresponding *in vitro* for 72 hours, and after stimulation.

In our system, however, neither of these mechanisms was likely because the APLs did not inhibit IL-17 production and cell proliferation with stimulation of $mGPI_{325-339}$, and induction of any anti-inflammatory and regulatory T cells was not detected. Nevertheless, it is probable that APLs inhibit $mGPI_{325-339}$-specific T cells because our analysis showed significant reductions of anti-mGPI Abs, which were Abs to thymus-dependent antigen [26]. We assumed that competitive bindings of APL to TCR *in vivo* were likely in our system; however, it cannot be denied that amino acid substitutions in peptides, even those that are not directly involved in MHC binding, might affect the overall structure of the peptide and biding affinity to MHC. Taken together, competitive binding of the APLs to $hGPI_{325-339}$-specific TCR or MHC *in vivo* is considered most likely as an inhibitory mechanism of APLs in our system.

The major interest in APLs is their clinical application; several studies showed that APLs suppress autoreactive cells in RA and Sjogren's syndrome [40,41]. Although clinical trials of APL in RA have not yet been conducted, phase II clinical trials in multiple sclerosis have been reported [42,43]. In one study

Figure 7



Co-immunization with altered peptide ligands suppresses production of antibodies to mouse glucose-6-phosphate isomerase. Sera were taken on day 28 from mice co-immunized with 10 μg glucose-6-phosphate isomerase $hGPI_{325-339}$ and 50 μg each altered peptide ligand (APL), and the titers of anti-mouse GPI IgG and IgG isotype were analyzed by ELISA. Each symbol represents a single mouse. Data presented as mean optimal density ± standard deviation. $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$ (Mann--Whitney U test).

of eight patients with multiple sclerosis, subcutaneous administration at 50 mg dose once-weekly of CGP77116 - an APL with substitutions at multiple TCR contact sites of MBP_{83-99} - resulted in the development of exacerbations in two patients with enhancement of MBP_{83-99}-reactive Th1 response [42]. Another double-blind placebo-controlled clinical trial included 142 patients who received various doses of subcutaneously injected NBI5788, an APL of MBP_{83-99} with substitutions at TCR contact sites [43]. In contrast to the former study, the administration of 5 mg APL weekly significantly decreased inflammatory lesions in the central nervous system. Unfortunately, the study was halted because 9% of the patients developed hypersensitivity reactions, but none discontinued at a dose of 5 mg in the double-blind phase whereas all patients discontinued in the double-blind phase after receiving higher doses of 20 or 50 mg. Low-dose APLs might therefore be useful agents for antigen-specific therapies of autoimmune diseases including RA, and their efficacy in RA might be more promising than in multiple sclerosis because drugs can be injected directly into the inflammatory lesions.

Finally, can GPI be a target of antigen-specific therapies in RA? It has been reported that patients with severe forms of RA retained high titers of anti-GPI Abs [44-47] and GPI-reactive $CD4^+$ T cells were detected among anti-GPI-Ab-positive patients with RA [48]. These findings highlight autoimmune responses to GPI are occurring in some patients with RA, and GPI can be a target of antigen-specific therapies to them - although further studies are needed to clarify the exact pathological role of GPI in RA.

Conclusions

The results of the present study showed that APLs with substitutions at TCR contact sites inhibit GPI peptide-induced arthritis. Novel antigen-specific therapies based on APLs may prove beneficial in arthritis induced by autoimmune responses to autoantigens.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KI wrote the manuscript and conducted all experiments. YY, AI, YK, KY YT, and RM assisted in the completion of the experiments. TS designed and coordinated the study. IM coordinated and directed the study. YN designed the APLs and provided advice for the study. TH, DG and SI participated in the discussion.

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Research article

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Tumor necrosis factor α-induced adipose-related protein expression in experimental arthritis and in rheumatoid arthritis

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Abstract

Introduction Tumor necrosis factor-alpha (TNFα) plays a pivotal role in rheumatoid arthritis (RA); however, the mechanism of action of TNFα antagonists in RA is poorly defined. Immunization of DBA/1 mice with glucose-6-phosphate isomerase (GPI) induces severe acute arthritis. This arthritis can be controlled by TNFα antagonists, suggesting similar etiology to RA. In this study, we explored TNFα-related mechanisms of arthritis.

Methods First, we performed GeneChip analysis using splenocytes of mice with GPI-induced arthritis. Expression of TNFα-induced adipose-related protein (TIARP) mRNA and protein in spleens, joints and lymph nodes was evaluated, and fluctuation of TIARP mRNA was analyzed after administration of anti-TNFα monoclonal antibody (mAb). Localization of TIARP in spleen and joints was also explored. Six-transmembrane epithelial antigen of the prostate (STEAP) families of proteins, the human ortholog of TIARP gene, were also evaluated in human peripheral blood mononucleocytes and synovium.

Results Among the arrayed TNFα-related genes, the expression of TIARP mRNA was the highest (more than 20 times the control). TIARP mRNA was detected specifically in joints and spleens of arthritic mice, and their levels in the synovia correlated with severity of joint swelling. Treatment with anti-TNF mAb significantly reduced TIARP mRNA expression in splenocytes. Among the splenocytes, $CD11b^+$ cells were the main source of TIARP mRNA. Immunohistochemistry showed that TIARP protein was mainly localized in hyperplastic synovium. Among the STEAP family of proteins, STEAP4 was highly upregulated in joints of patients with RA and especially co-localized with $CD68^+$ macrophages.

Conclusions The results shed light on the new mechanism of action of TNFα antagonists in autoimmune arthritis, suggesting that TIARP plays an important role in inflammatory arthritis, through the regulation of inflammatory cytokines.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder with a variable disease outcome and is characterized by inflammation of multiple joints. The prognosis of RA patients has improved significantly in recent years after the introduction of tumor necrosis factor-alpha (TNFα)-based therapy [1]. Despite the wide use of these biologics, their precise mechanisms of action in RA remain unclear.

Several animal models of RA have been described; however, the therapeutic benefits of TNF antagonists have been confirmed in only a few of these models. Schubert and colleagues

CFA: complete Freund's adjuvant; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; GEO: Gene Expression Omnibus; GPI: glucose-6-phosphate isomerase; GST: glutathione S-transferase; HRP: horseradish peroxidase; IL-6: interleukin-6; mAb: monoclonal antibody; MACS: magnetic-activated cell sorting; MW: molecular weight; OA: osteoarthritis; PBMC: peripheral blood mononuclear cell; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; RA: rheumatoid arthritis; STEAP: six-transmembrane epithelial antigen of the prostate; TIARP: tumor necrosis factor alpha-induced adipose-related protein; TNF: tumor necrosis factor; TNFR: tumor necrosis factor receptor.

[2] reported that continuous injections of human TNF receptor (TNFR) p75-IgG-Fc fusion protein (Etanercept) from days 0 to 9 completely protected against the development of arthritis in glucose-6-phosphate isomerase (GPI)-induced arthritis. In this regard, we recently demonstrated a clear therapeutic effect of anti-TNF monoclonal antibody (mAb) in mice with GPI-induced arthritis, and the therapeutic response correlated with the *in vitro* regulation of TNF production [3]. We also identified that anti-interleukin-6 (IL-6) receptor mAb blocks the development of GPI-induced arthritis [3,4]. These results indicate that the GPI-induced arthritis model is suitable for studying the mechanisms of action of TNFα antagonists as well as IL-6 antagonists in RA patients.

Using such a TNFα-dependent arthritis model, we investigated TNFα-related molecules by GeneChip analysis. The expression of TNFα-induced adipose-related protein (TIARP) was the highest in GeneChip study. TIARP was identified as a transmembrane protein that is highly regulated by TNFα in adipocytes [5]. Not only TNFα but also IL-6 regulated the expression of TIARP [6], suggesting the involvement of the inflammatory cascade in RA. To our knowledge, however, no information on its role in arthritis or its localization in joints has been published.

To explore the role of TIARP in arthritis, we conducted the present study in GPI-induced arthritis. TIARP mRNA and proteins were upregulated in joints and spleens in mice with GPI-induced arthritis. Administration of anti-TNFα mAb reduced TIARP mRNA in splenocytes. In arthritic mice, TIARP mRNA was expressed mainly in $CD11b^+$ cells in the spleen, and TIARP mRNA level was increased in the joints (accompanied by joint swelling), especially in hyperplastic synovium. Overexpression of the human TIARP counterpart, such as six-transmembrane epithelial antigen of the prostate-4 (STEAP4), was noted in the synovia of patients with RA. The results provide the first characterization of the role of TIARP in inflammatory arthritis.

Materials and methods

Glucose-6-phosphate isomerase-induced arthritis

Male DBA/1 mice (6 to 8 weeks old) were obtained from Charles River Laboratories (Yokohama, Japan). Recombinant human GPI was prepared as described previously [7]. Mice were immunized by intradermal injection of 300 μg of recombinant human GPI-GST (glutathione S-transferase) (hGPI) in emulsified complete Freund's adjuvant (CFA) (Difco Laboratories Inc., now part of Becton Dickinson and Company, Franklin Lakes, NJ, USA). Control mice were immunized with 100 μg of GST in CFA. Arthritic animals were assessed visually, and changes in each paw were scored on a scale of 0 to 3. A score of 0 indicates no evidence of inflammation, 1 indicates subtle inflammation or localized edema, 2 indicates swelling that is easily identified but localized to the dorsal or ventral surface of paws, and 3 indicates swelling on all aspects of paws, and the maximum possible score was 12 per mouse. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the University of Tsukuba (Japan).

GeneChip analysis of splenocytes from glucose-6-phosphate isomerase-induced arthritis

The spleens of three GPI-GST (molecular weight [MW] = 89 kDa) (300 μg)-immunized DBA/1 mice were harvested on day 10. As a control, the spleens of three GST (MW = 26 kDa) (100 μg)-immunized DBA/1 mice were used. Total RNA was extracted from the splenocytes using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan), and then 15 μg of RNA was used for cDNA synthesis by reverse transcription followed by synthesis of biotinylated cRNA through *in vitro* transcription. After cRNA fragmentation, hybridization with mouse 430A2.0 GeneChip (Affymetrix, Santa Clara, CA, USA) with probes for 43,000 mouse gene ESTs (expressed sequence tags) was performed in accordance with the protocol provided by the manufacturer. Analysis was performed by gene expression software.

Analysis of TIARP and tumor necrosis factor-alpha gene expression

Spleens and lymph nodes were isolated, cut into small pieces, and passed through cell strainers (BD Biosciences, Erembodegem, Belgium) to obtain single-cell suspensions. The remaining cells were washed twice with phosphate-buffered saline (PBS). Synovial tissues from the ankle joints were isolated and minced by scissors. Total RNA was extracted with ISOGEN in accordance with the instructions provided by the manufacturer. cDNA was obtained by reverse transcription with a commercially available kit (Fermentas, Glen Burnie, MD, USA). Primers sequenced were as follows: TIARP sense 5'-AGCCCACGTGGTCAAAGCAT-3' and antisense 5'-CCTTGGTCCAGTGGGGTGA-3' and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) sense 5'-CGTCCCGTAGACAAAATGGT-3' and antisense 5'-GAATTTGCCGTGAGTGGAGT-3'.

All polymerase chain reactions (PCRs) were performed in a Takara PCR Thermal Cycler (Takara Bio Inc., Shiga, Japan). After denaturation at 95°C for 5 minutes, cycles were set at 10 seconds at 94°C, 10 seconds at 60°C, and 30 seconds at 72°C. Cycling was followed by 10 minutes of elongation at 72°C. PCR products were subjected to electrophoresis in 1% agarose gels in Tris-borate-EDTA (ethylenediaminetetraacetic acid) electrophoresis buffer, stained with ethidium bromide, and detected by ultraviolet transillumination. cDNA samples were normalized for the housekeeping gene *GAPDH*.

For real-time PCR, we used a TaqMan Assay-on-Demand gene expression product (Applied Biosystems, Foster City, CA, USA). The expression levels of TIARP, TNFα, and GAPDH (assay ID Mm00475402_m1, Mm00443258_m1, and Mm99999915_g1, respectively; Applied Biosystems) were

normalized relative to the expression of GAPDH. Analysis was performed with an ABI Prism 7500 apparatus (Applied Biosystems) under the following conditions: inactivation of possible contaminating amplicons with AmpErase UNG for 2 minutes at 50°C, initial denaturation for 10 minutes at 95°C, followed by 45 thermal cycles of 15 seconds at 95°C and 60 seconds at 60°C. The serum TNFα level was measured by an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Inc., San Diego, CA, USA). After conditioning, the detection limit of TNFα concentration was 2 μg/mL.

Preparation of anti-TIARP and anti-STEAP4 antibodies

One rabbit was immunized subcutaneously by TIARP peptide$_{5-19}$ (HADEFPLTTDSSEKQ, amino-terminal peptide coupled to keyhole limpet hemocyanin) or human ortholog STEAP4 peptide$_{3-15}$ (KTCIDALPLTMNS) [8] with CFA four times, on days 0, 14, 28, and 42. The rabbit was sacrificed on day 52, and serum was collected. Serum was first purified by protein A column and then affinity-purified by TIARP-peptide$_{5-19}$ or STEAP4 peptide$_{3-15}$ column. The purified fraction was confirmed by TIARP peptide$_{5-19}$ or STEAP4 peptide$_{3-15}$ ELISA.

Western blotting

The cells were washed with PBS and incubated with lysis buffer (pH 7.4, 50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5% NP-40). Where indicated, protein concentrations were quantified using the bicinchoninic acid reagent (Pierce, Rockford, IL, USA). Samples (10 μg of total protein) were separated by SDS-PAGE (4/20% acrylamide; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All subsequent wash buffers contained 0.05% Tween-20 in PBS. Four percent Block Ace (Dainippon Pharmaceutical, Osaka, Japan) was used to block the membranes and to dilute antibodies. Rabbit polyclonal anti-TIARP antibodies and rabbit anti-actin antibodies (Sigma-Aldrich, Munich, Germany) were used at 1:3,000 dilution. Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:6,000 dilution; Bio-Rad Laboratories, Inc.) were used to visualize bound anti-TIARP antibodies or anti-actin antibodies with the ECL [enhanced chemiluminescence] Western blot detection kit (Amersham, now part of GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Treatment with anti-tumor necrosis factor-alpha monoclonalantibody

We used commercially available anti-TNFα mAb (eBioscience, Inc.). For a control antibody, we used similar amounts of rat IgG1 isotype control (R&D Systems, Inc., Minneapolis, MN, USA). Just after the onset of arthritis (on day 8), a single dose of 100 μg of anti-TNFα mAb or control antibody was injected. Spleen was harvested at the indicated time points and analyzed for TIARP expression. Three independent experiments were performed.

Identification of TIARP-positive cells in splenocytes of mice with glucose-6-phosphate isomerase-induced arthritis

The spleens were harvested on day 12 after GPI immunization and single-splenocyte cell suspensions were prepared as described above. CD4$^+$, CD19$^+$, CD11b$^+$, and CD11c$^+$ cells from splenocytes were isolated by magnetic beads using the MACS™ [magnetic-activated cell sorting] system (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells contained more than 97% CD4$^+$, CD19$^+$, CD11b$^+$, and CD11c$^+$ cells as confirmed by fluorescence-activated cell sorting analysis. The cells were dispensed at 1×10^6 cells to analyze the expression of TIARP mRNA.

Immunohistochemical staining for TIARP/STEAP4

At the indicated time points, the ankles of the mice were removed, fixed, decalcified, and paraffin-embedded. Sections (5-μm thick) were stained with hematoxylin and eosin and were evaluated for histological changes. For immunohistochemical study, endogenous peroxidase activity was inhibited using 3% hydrogen peroxidase in methanol. Sections were blocked by 5% bovine serum albumin in PBS for 10 minutes and then incubated with rabbit anti-TIARP antibody (1:100 dilution) or normal rabbit Ig (1:100 dilution; Dako, Tokyo, Japan). Isotype-matched HRP-conjugated anti-rabbit IgG antibody (Bio-Rad Laboratories, Inc.) was added for 30 minutes. HRP activity was detected using 3,3-diaminobendine (DAB) (Nichirei Corporation, Tokyo, Japan) as a substrate. The stained sections were counterstained with Mayer's hematoxylin for 10 seconds and mounted with aqueous mounting medium.

For human STEAP4 staining, synovial tissues were obtained after informed consent was given by RA patients at the time of joint replacement. All RA patients satisfied the classification criteria of the American College of Rheumatology (1987) [9]. The synovium was embedded in optimal cutting temperature compound and frozen in dry ice isopentane, and 5-μm-thick sections were mounted at -25°C. Anti-human STEAP4 polyclonal antibody conjugated with fluorescein isothiocyanate (FITC protein labeling kit; Pierce) and purified anti-human CD68 (BD Pharmingen, San Diego, CA, USA) conjugated with rhodamine (1:100 dilution, Rhodamine protein labeling kit; Pierce) were used. Nuclei were counterstained with 4'-6'-diamidine-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Inc., now part of Invitrogen Corporation, Carlsbad, CA, USA). The stained sections were examined under a fluorescent microscope (model FW4000; Leica Microsystems, Tokyo, Japan).

Patients and analysis of human peripheral blood mononuclear cells and synovium for STEAP proteins

Peripheral blood mononuclear cells (PBMCs) from three female patients with RA and three healthy control subjects were obtained. All RA patients satisfied the classification criteria of the American College of Rheumatology (1987) [9]. Synovial tissues from 36 RA and 19 osteoarthritis (OA) patients were obtained at the time of total knee replacement. Written informed consent was obtained from all subjects, and the study was approved by the ethics review committee. Total RNA was extracted with ISOGEN in accordance with the protocol provided by the manufacturer. cDNA was obtained by reverse transcription with a commercially available kit. The following primers were used: STEAP2 sense 5'-CCTACAGCCTCTGCTTACCG-3' and antisense 5'-GAGGGCAAAACAAGAGCAAG-3', STEAP3 sense 5'-GCCAGAAGAGATGGACAAGC-3' and antisense 5'-GGTGCTCTTGCTCTGTAGGG-3', STEAP4 sense 5'-GCTCTCCAGTCAGGAGCACT-3' and antisense 5'-CACACAGCACAGCAGACAAA-3', and GAPDH sense 5'-GAAGGTGAAGGTCGGAGTC-3' and antisense 5'-GAAGATGGTGATGGGATTTC-3'. For real-time PCR, we used a TaqMan Assay-on-Demand gene expression product (Applied Biosystems). The expression level of STEAP4 was normalized relative to the expression of GAPDH. Methods were described above.

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 of less than 0.05 denoted the presence of a statistically significant difference.

Results

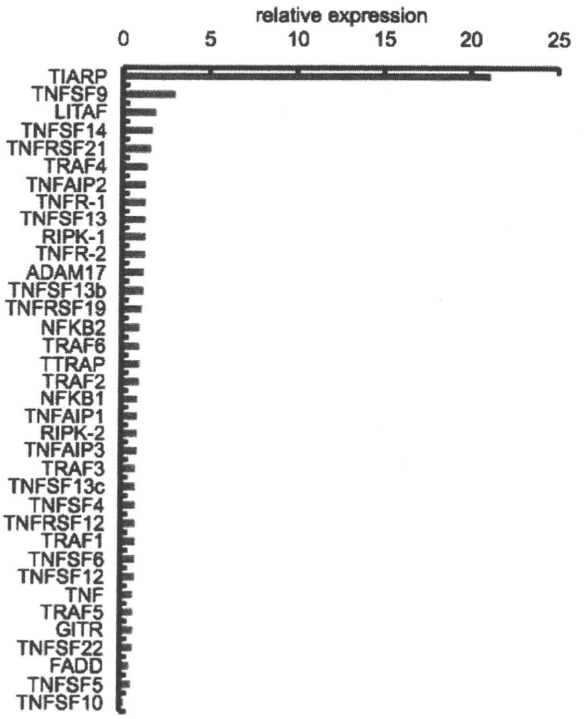
Induction of glucose-6-phosphate isomerase-induced arthritis

DBA/1 mice were immunized using the human recombinant GPI as reported previously [3,4]. All mice developed arthritis after immunization with 300 μg of GPI. Arthritis was documented at day 8, and severe arthritis was recorded at day 14, with ankle swelling reaching a maximum at day 14 but subsiding gradually on follow-up.

Overexpression of tumor necrosis factor-induced adipose-related protein in splenocytes of arthritic mice

To explore TNF-related genes in GPI-induced arthritis, we performed GeneChip analysis using arthritic splenocytes and control-immunized splenocytes. Among the arrayed TNFα-related genes, TIARP mRNA was highly expressed in arthritic splenocytes, with levels exceeding more than 20 times those of the control splenocytes (Figure 1). This finding suggests that TIARP protein is an important molecule in TNFα-dependent arthritis. The data discussed in this publication have been deposited in the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (Bethesda, MD, USA) and are accessible through GEO Series accession number [GEO:GSE17272] [10].

Figure 1



Upregulation of tumor necrosis factor-alpha (TNFα)-related genes in splenocytes of mice with glucose-6-phosphate isomerase (GPI)-induced arthritis. The mRNA expression levels of TNF-related genes in splenocytes of mice with GPI-induced arthritic (at day 10) relative to control splenocytes are shown. TNFα-induced adipose-related protein (TIARP) was specifically and strongly induced in splenocytes. GeneChip analysis was performed by gene expression software. ADAM17, a disintegrin and metallopeptidase domain 17; FADD, Fas (tumor necrosis factor receptor superfamily 6)-associated via death domain; GITR, glucocorticoid-induced tumor necrosis factor-related protein-D mRNA; LITAF, lipopolysaccharide-induced tumor necrosis factor-alpha factor; NFKB1, nuclear factor kappa B subunit p105; NFKB2, nuclear factor kappa B subunit p100; RIPK, receptor (tumor necrosis factor receptor superfamily)-interacting serine-threonine kinase 1 and 2; TNFAIP, tumor necrosis factor alpha-induced protein; TNFR, tumor necrosis factor receptor; TNFRSF, tumor necrosis factor receptor superfamily; TNFRSF12, WSL-1-like protein; TNFRSF22, tumor necrosis factor receptor family member SOBa mRNA; TNFSF, tumor necrosis factor (ligand) superfamily; TRAF, tumor necrosis factor receptor-associated factor; TTRAP, tumor necrosis factor receptor-associated factor and tumor necrosis factor receptor-associated protein.

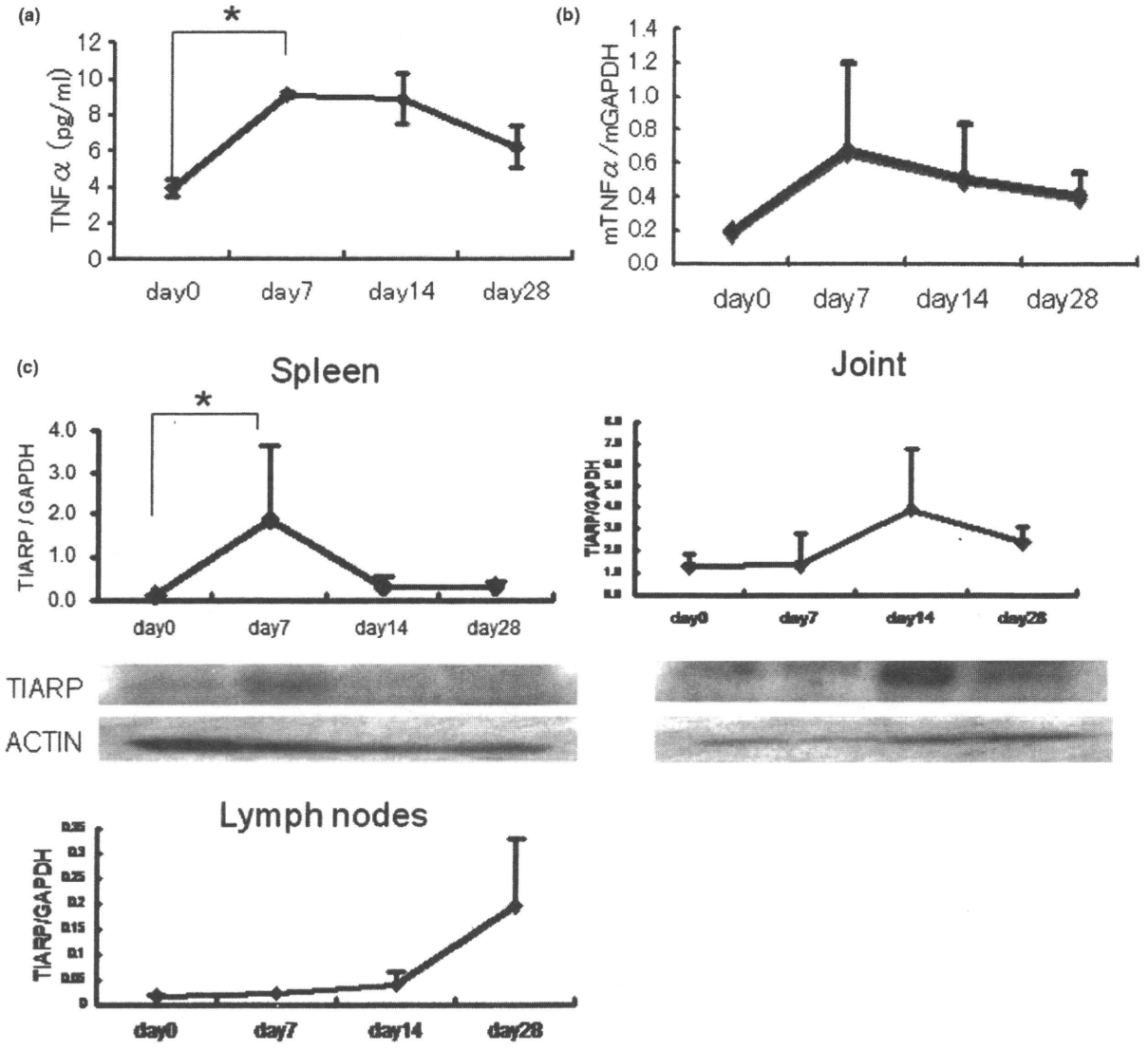
Tumor necrosis factor-alpha and TIARP expression in glucose-6-phosphate isomerase-induced arthritis

To determine the correlation between TNFα and TIARP in GPI-induced arthritis, the time course of TIARP expression was analyzed. Serum TNFα levels were elevated at day 7

(onset of arthritis, $P < 0.05$), were at the same elevated levels at day 14 (peak of arthritis), and then subsided to the basal level at day 28 (Figure 2a). In contrast, the TNFα mRNA expression level in arthritic joints tended to increase at day 7, though insignificantly, in mice with GPI-induced arthritis. The expression level decreased later to basal levels (Figure 2b).

Both real-time PCR and Western blotting showed upregulation of TIARP mRNA and protein expression at day 7 in splenocytes of mice with GPI-induced arthritis (Figure 2c, left panel). In the joints of the same mice, upregulation of TIARP mRNA and protein was noted at days 14 and 28, and the expression correlated with joint swelling (Figure 2c, right

Figure 2



Serial changes in expression levels of tumor necrosis factor-alpha (TNFα) and TIARP in glucose-6-phosphate isomerase (GPI)-induced arthritis. Serial changes in TNFα concentrations in **(a)** serum and **(b)** arthritic joints and **(c)** TIARP mRNA and protein expression in spleens (left and middle panels) and arthritic joints (right panel) by real-time polymerase chain reaction (PCR) and Western blotting in mice with GPI-induced arthritis. As shown in the bottom panel of **(c)**, TIARP mRNA in lymph nodes was also analyzed. Arthritis appeared on days 7 and 8, peaked in severity on day 14, and then gradually subsided. High expression levels of TIARP mRNA and proteins were detected in splenocytes on day 7 (the onset of arthritis). In joints, the expression of TIARP mRNA and protein was correlated with joint swelling (days 14 and 28). 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; mTNFα, murine tumor necrosis factor-alpha; TIARP, tumor necrosis factor alpha-induced adipose-related protein.