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Research article

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

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Abstract

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

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

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

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

Introduction

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

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

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

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

Materials and methods

Mice

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

GPI and synthetic peptides

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

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

Induction of arthritis

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

Treatments of arthritis with anti-IL-17 monoclonal antibodies

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

Analysis of cytokine production

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

Intracellular cytokine staining and flow cytometric analysis

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

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

Analysis of anti-GPI antibody

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

Immunohistology

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

Statistical analysis

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

Results

I-A^q binding motif and epitope candidates

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

Epitope screening

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

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

Table 1

I-Aq binding motifs

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

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

Table 2

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

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

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

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

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

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

Peptide-induced arthritis is mediated by Th17

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

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

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

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

Table 3

Synthetic peptides for screening T cell epitopes

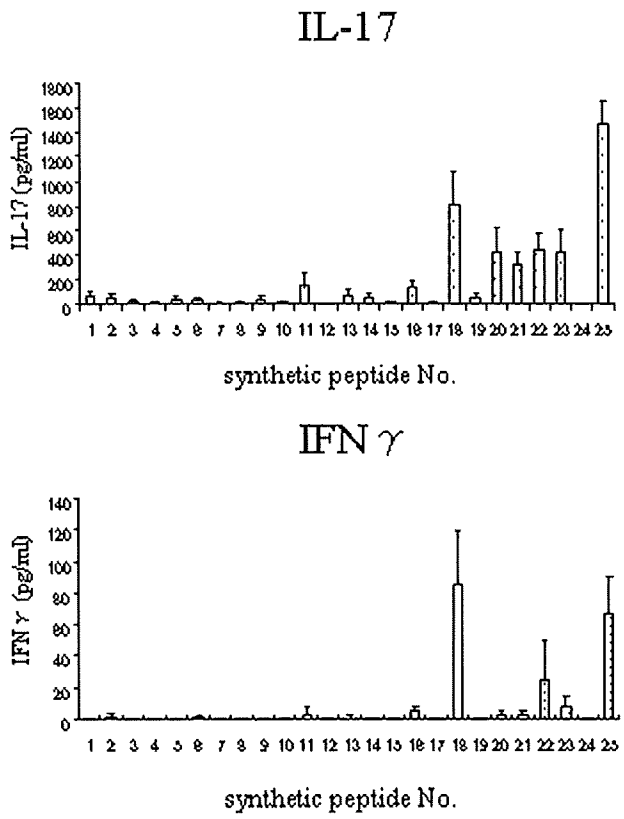
Peptide number	Peptide	Synthetic peptide sequence
1	1–20	H-MAALTRDPQFQKLQQWYREH-OH
2	23–42	H-ELNLRRLFDANKDRFNHFSL-OH
3	37–56	H-FNHFSLTLNTNHGHILVDYS-OH
4	51–70	H-ILVDYSKNLVTEDVMRMLVD-OH
5	71–90	H-LAKSRGVEAARERMFNGEKI-OH
6	96–115	H-RAVLHVALRNRSNTPILVDG-OH
7	145–164	H-TGKTITDVINIGIGGSDLGP-OH
8	162–181	H-LGPLMVTEALKPYSSGGPRV-OH
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



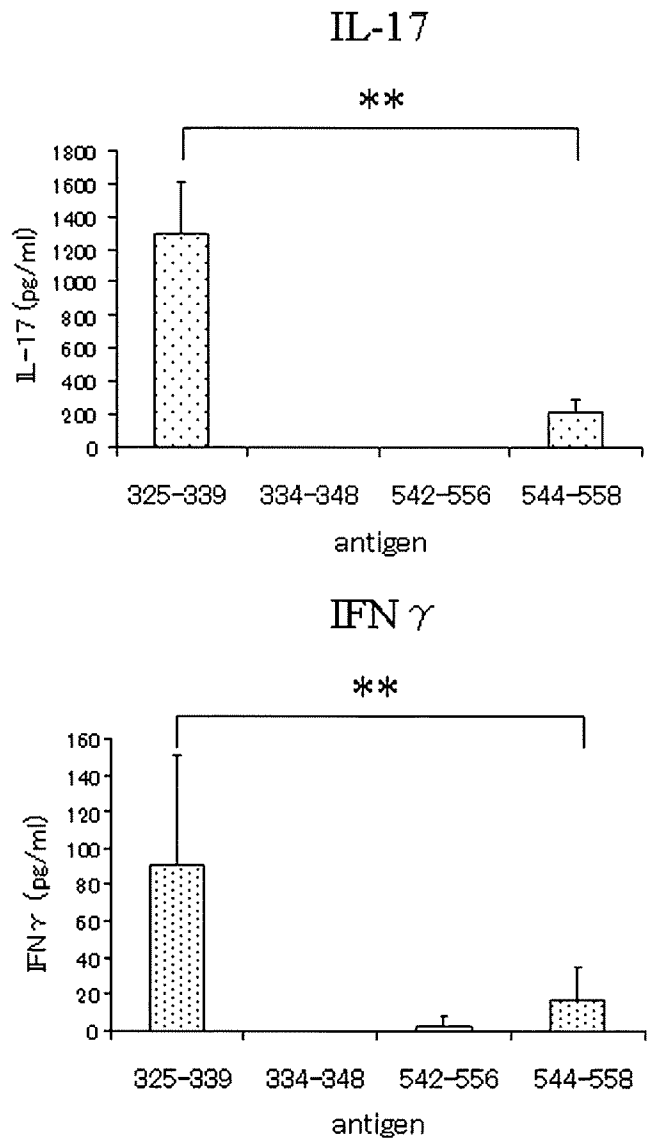
Synthetic peptides number 18 and 25 produced marked simulation of glucose-6-phosphate isomerase (GPI) primed CD4+ T cells. Mice were sacrificed on day 7 after immunisation. CD4+ T cells were purified from spleen cells of GPI-immunised DBA/1 mice. GPI-primed CD4+ T cells and antigen presenting cells (APCs) 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.

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

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

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

Figure 2



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

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

Table 4

Re-synthesised peptides used for determining a major epitope

Peptide number	Peptide	Synthetic peptide sequence
18	327–346	H-Y**I**N**C**F**G**C**E**T**H**A**M**L**P**Y**D**Q**Y**LH-OH
	325–339	H-IWY**I**N**C**F**G**C**E**TH**A**ML-OH
	334–348	H-ETH**A**M**L**P**Y**D**Q**Y**L**HRF-OH
25	539–558	H-DASTNG**L**IN**F**I**K**Q**Q**REARVQ-OH
	542–556	H-TNG**L**IN**F**I**K**Q**Q**REAR-OH
	544–558	H-G**L**IN**F**I**K**Q**Q**REARVQ-OH

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

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

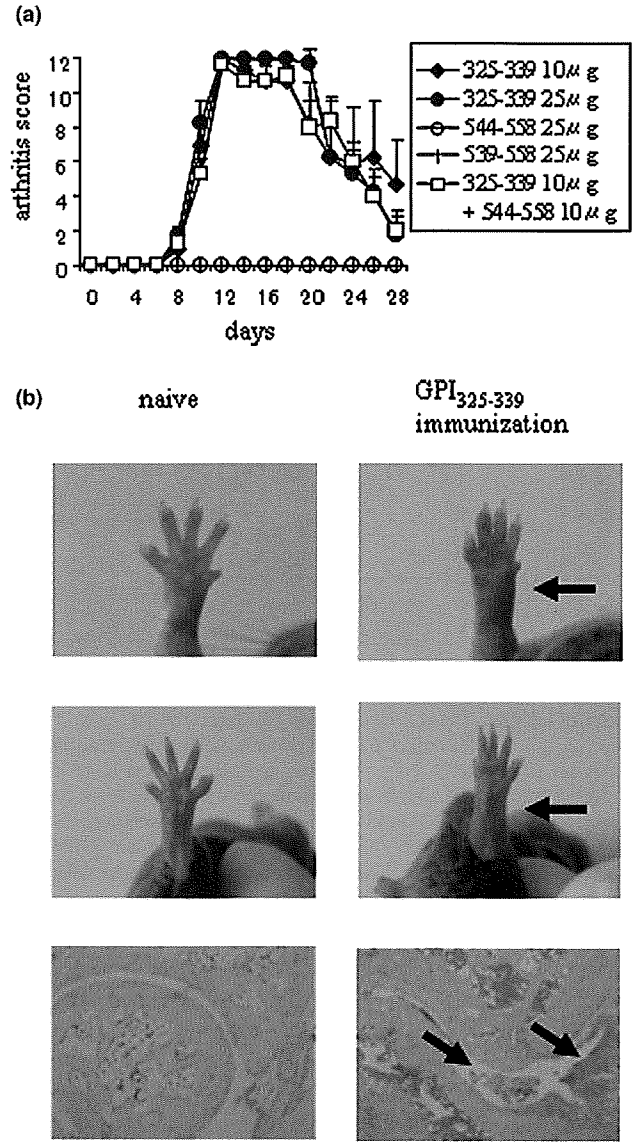
Next, we investigated the existence of IgG on the cartilage surface by immunohistology, because GPI were proved to deposit on the cartilage surface of normal naïve mice [23]. The cryostat sections of ankle joints from naïve mice and mice immunised with $hGPI_{544-558}$ did not show IgG deposit on the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

Discussion

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

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

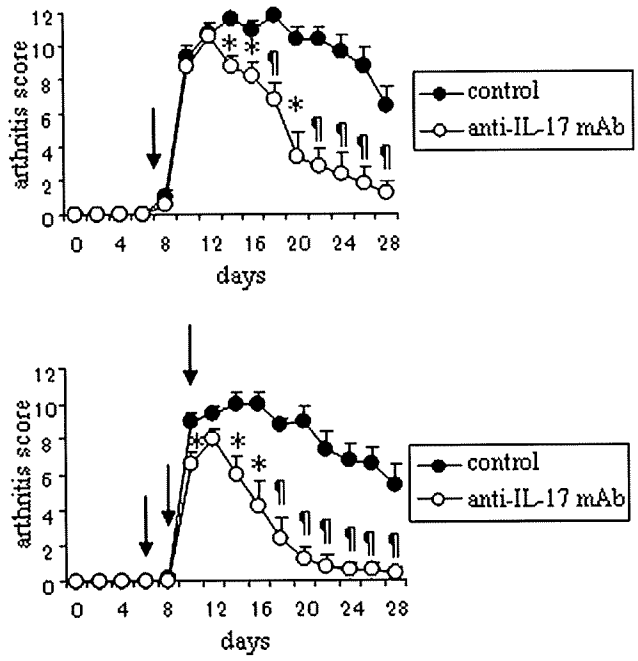
Figure 3



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

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

Figure 4



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

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

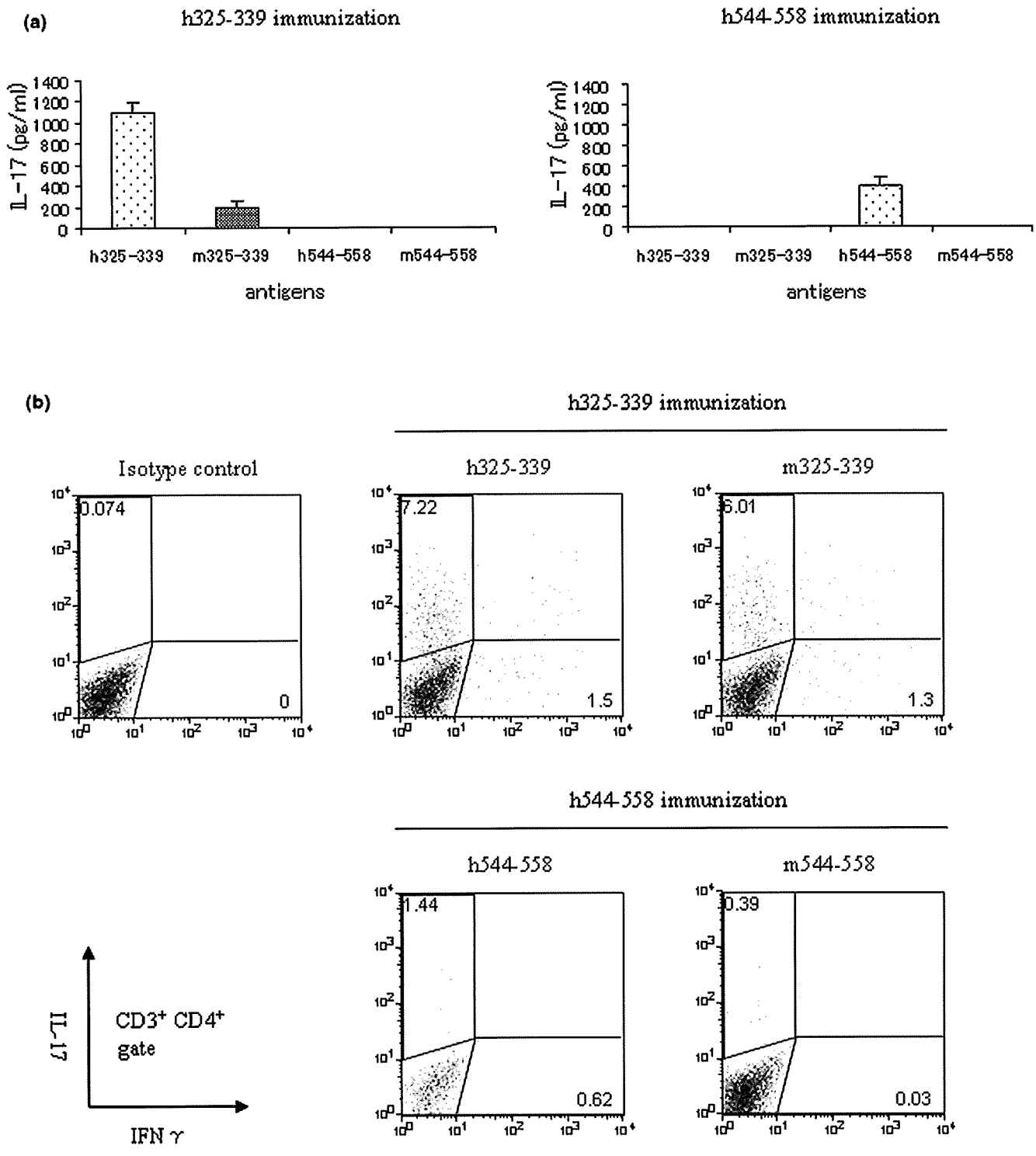
Our study demonstrated that immunisation with $hGPI_{325-339}$ induced antigen-specific Th17 cells, which can cross-react with $mGPI_{325-339}$ and lead B cells to produce anti-mouse GPI antibodies. However, immunisation with $hGPI_{544-558}$ could not even induce $hGPI_{544-558}$-specific Th17 cells.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

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

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

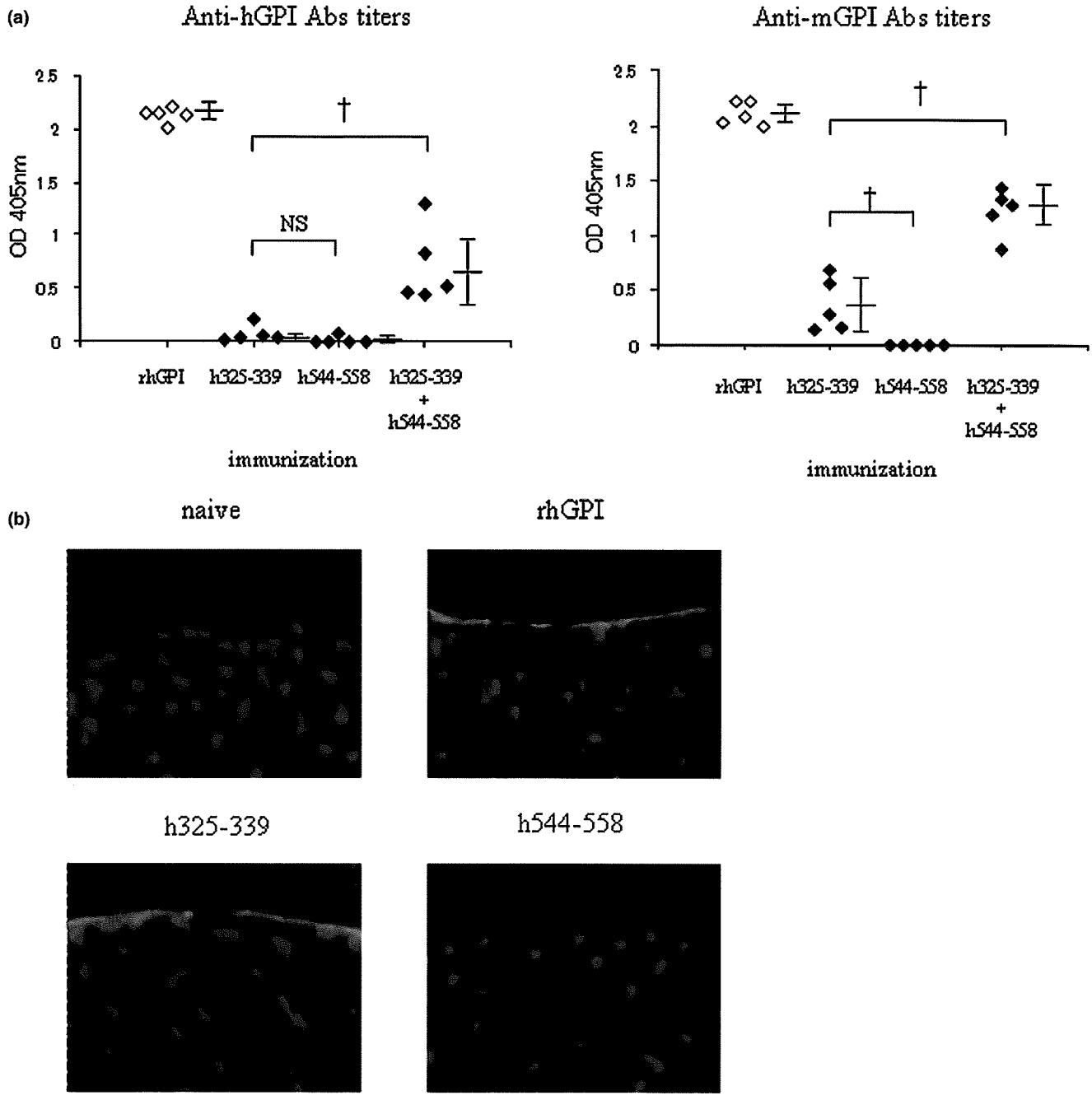
From a probability point of view, it is possible that other epitopes exist in some regions of human GPI-amino acid

Figure 5



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

Figure 6



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

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

Table 5

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

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

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

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

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

Conclusions

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

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

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

Acknowledgements

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nature immunology

Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5

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The intestinal cell types responsible for defense against pathogenic organisms remain incompletely characterized. Here we identify a subset of CD11c^{hi}CD11b^{hi} lamina propria dendritic cells (LPDCs) that expressed Toll-like receptor 5 (TLR5) in the small intestine. When stimulated by the TLR5 ligand flagellin, TLR5$^+$ LPDCs induced the differentiation of naive B cells into immunoglobulin A–producing plasma cells by a mechanism independent of gut-associated lymphoid tissue. In addition, by a mechanism dependent on TLR5 stimulation, these LPDCs promoted the differentiation of antigen-specific interleukin 17–producing T helper cells and type 1 T helper cells. Unlike spleen DCs, the LPDCs specifically produced retinoic acid, which, in a dose-dependent way, supported the generation and retention of immunoglobulin A–producing cells in the lamina propria and positively regulated the differentiation interleukin 17–producing T helper cells. Our findings demonstrate unique properties of LPDCs and the importance of TLR5 for adaptive immunity in the intestine.

The gastrointestinal tract is constantly exposed to food proteins and commensal bacteria. Although the intestinal immune system has evolved mechanisms to maintain immunological tolerance to food and commensal organisms, it also recognizes invasive pathogens and properly induces protective immune responses to eliminate them. Dendritic cells (DCs) are thought to be critical in the 'decision' of whether to mount tolerant or protective immune responses[1]. Many subsets of DCs have been identified in the intestine[2]. In the Peyer's patches and mesenteric lymph nodes, conventional DCs consist of CD11c^{hi}CD11b$^+$CD8α^-, CD11c^{hi}CD11b$^-$CD8α^+ and CD11c^{hi}CD11b$^-$CD8α^- subsets[2]. In addition, there are CD11c^{int} plasmacytoid DCs in these sites[3,4]. Peyer's patch DCs produce interleukin 10 (IL-10) rather than IL-12, polarize naive T cells toward T helper type 2 (T_H2) or regulatory phenotypes[5] and induce the differentiation of plasma cells positive for immunoglobulin A (IgA)[6,7].

In contrast, lamina propria DCs (LPDCs) are less well studied. Although DCs are dominant antigen-presenting cells in the small intestine, colonic DCs are concentrated mainly in isolated lymphoid follicles, few of which are present in the lamina propria[8]. However, studies have shown that LPDCs of the small intestine and DCs in mesenteric lymph nodes that express CD103 have regulatory functions[9,10]. CD103$^+$ LPDCs migrate from the lamina propria to the mesenteric lymph nodes in a CCR7-dependent way[11–13] and promote the generation of Foxp3$^+$ regulatory T cells by means of retinoic acid[14]. Subsequent studies, however, have shown that CD11b$^+$F4/80$^+$CD11c$^-$ macrophages in the lamina propria are more potent inducers of regulatory T cells than are LPDCs and that CD11b$^+$ LPDCs generate T cells producing IL-17 *in vitro*[15]. These findings collectively suggest that LPDCs induce both 'tolerogenic' regulatory T cells and 'inflammatory' IL-17–producing T helper cells (T_H-17 cells). However, it remains unclear what kind of stimulation triggers the LPDC-induced generation of T_H-17 cells.

The Toll-like receptor (TLR) family, which is key for innate immunity, consists of 13 mammalian members[16]. TLRs are 'preferentially' expressed in 'professional' antigen-presenting cells such as DCs and macrophages and recognize specific components of

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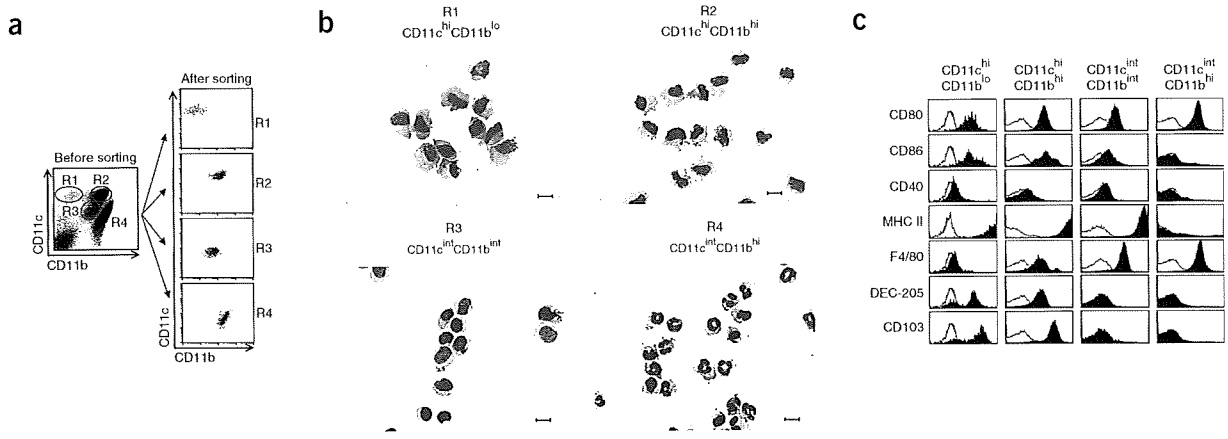

Figure 1 Four subsets of CD11c$^+$ LPCs in the small intestine. (**a**) Flow cytometry of intestinal low-density LPCs stained for CD11b and CD11c, before and after sorting. (**b**) May-Grunwald-Giemsa staining of four leukocyte subsets (gated in **a**) from the lamina propria. Scale bars, 10 μm. (**c**) Surface expression of CD80, CD86, CD40, major histocompatibility complex class II (MHC II), F4/80, DEC-205 and CD103 (filled histograms) on the four leukocyte subsets gated in **a**. Open histograms, isotype control. Data are representative of at least three independent experiments.

microorganisms to induce innate immune responses[16]. Each TLR activates specific signaling pathways that elicit biological responses to microorganisms, as well as DC maturation and cytokine production that shape adaptive immune responses[16]. Although the function of TLRs has been examined extensively in intestinal epithelial cells[17], the function of TLRs in lamina propria antigen-presenting cells has not been fully elucidated. Intestinal CD11c$^+$ lamina propria cells (LPCs) have high expression of TLR5 (A002297) and induce inflammatory responses when stimulated with the TLR5 ligand bacterial flagellin[18]. Unlike conventional DCs, such as those in the spleen (SPDCs), CD11c$^+$ LPCs do not express TLR4, which recognizes the Gram-negative bacterial component lipopolysaccharide (LPS)[18]. Nevertheless, *Tlr5*$^{-/-}$ mice show resistance to oral *Salmonella typhimurium* infection, as this facultative intracellular flagellated bacteria seems to use TLR5 and CD11c$^+$ LPCs as 'carriers' for systemic infection[18].

Mouse CD11c$^+$ LPCs consist of four subsets distinguished by differential expression patterns of CD11c and CD11b. Here we have identified a subset of CD11c^{hi}CD11b^{hi} LPDCs as TLR5-expressing cells. In response to flagellin, these LPDCs induced the differentiation of naive B cells into IgA$^+$ (A001174) plasma cells by a mechanism independent of gut-associated lymphoid tissue (GALT) and triggered the differentiation of antigen-specific T_H-17 and T_H1 cells. In a dose-dependent way, retinoic acid produced by LPDCs supported the generation and retention of IgA-producing cells in the lamina propria and positively regulated T_H-17 cell differentiation.

RESULTS

High TLR5 expression on CD11c^{hi}CD11b^{hi} LPDCs

CD11c$^+$ DCs constituted 10–15% of leukocytes in the small intestinal lamina propria and consisted of at least two subsets (CD11c^{hi}CD11b^{lo} (R1) and CD11c^{hi}CD11b^{hi} (R2))[12] (**Fig. 1a,b**), each of which had a DEC-205$^+$ major histocompatibility complex class II–high CD80$^+$CD86$^+$CD103$^+$ surface phenotype (**Fig. 1c**). In addition, CD11c^{hi}CD11b^{hi} cells had moderate expression of F4/80, which indicated a macrophage-like character. The remaining CD11c$^+$ subsets consisted of CD11c^{int}CD11b^{int} cells (R3), which are F4/80$^+$DEC-205$^-$ major histocompatibility complex class II$^+$ phagocytic macrophages[15,19], and CD11c^{int}CD11b^{hi} cells (R4), which are eosinophils with uniquely shaped nuclei and eosinophilic granules[12] (**Fig. 1b,c**). Of these four subsets from the lamina propria of C57BL/6 mice, only CD11c^{hi}CD11b^{hi} LPDCs expressed *Tlr5* mRNA (**Fig. 2a**). Consistent with the expression of functional TLR5, CD11c^{hi}CD11b^{hi} LPDCs produced proinflammatory cytokines such as IL-6, IL-12p40 and IL-12p70, but not IL-23 or IL-10, in response to flagellin (**Fig. 2b**). In contrast, LPDCs (R1) did not produce such cytokines in response to either flagellin or LPS (**Supplementary Fig. 1** online). Thus, CD11c^{hi}CD11b^{hi} LPDCs are responsible for TLR5-mediated innate immune responses.

CD11c^{hi}CD11b^{hi} LPDCs induce IgA production

To determine the function of CD11c^{hi}CD11b^{hi} LPDCs in adaptive immunity, we examined IgA synthesis in the small intestine. IgA is the

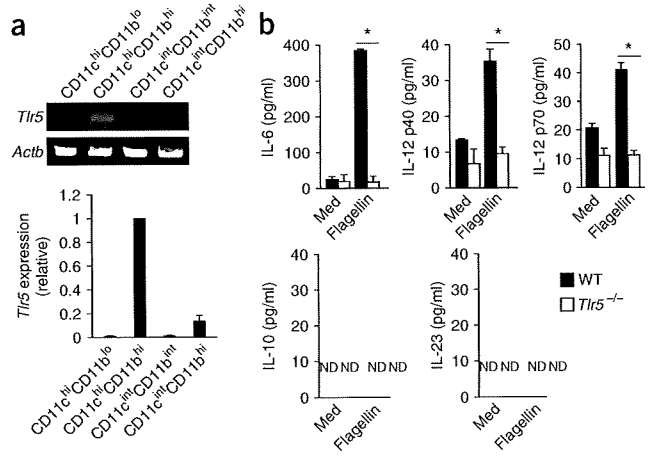

Figure 2 CD11c^{hi}CD11b^{hi} LPDCs specifically express TLR5. (**a**) RT-PCR (top) and quantitative real-time PCR (bottom) of *Tlr5* expression in the four leukocyte lamina propria subsets. *Actb* encodes β-actin (top, loading control). Expression (bottom) is relative to that of *Actb*. Data are representative of three independent experiments. (**b**) Cytokine production by CD11c^{hi}CD11b^{hi} LPDCs from wild-type (WT) and *Tlr5*$^{-/-}$ mice in response to medium alone (Med) or flagellin (1 μg/ml). ND, not detected. *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments.

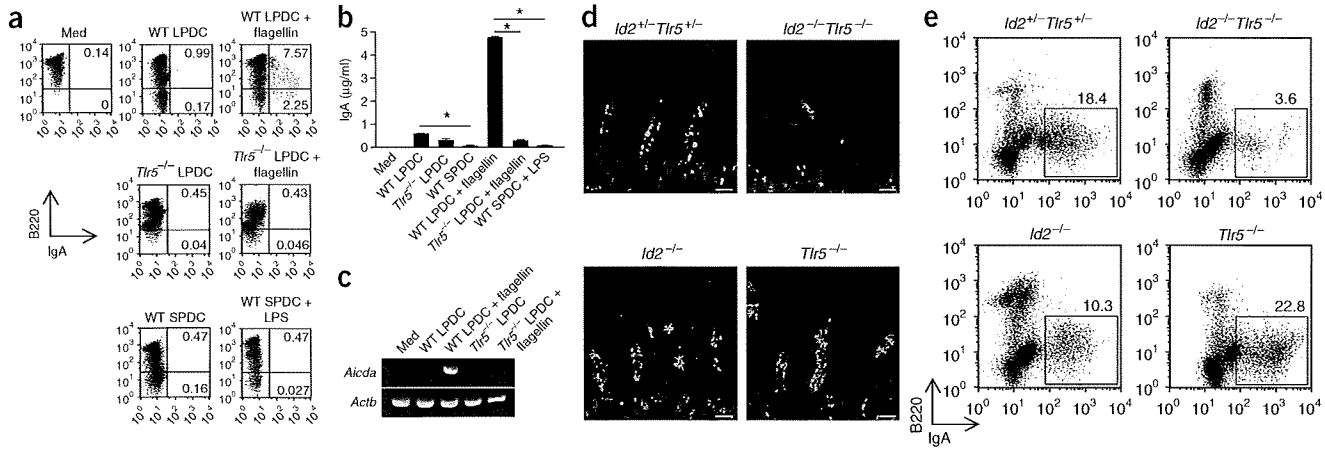

Figure 3 CD11c^{hi}CD11b^{hi} LPDCs induce IgA$^+$ plasma cell differentiation. (**a**,**b**) Flow cytometry (**a**) and ELISA (**b**) of peritoneal IgM$^+$IgD$^+$ cells cultured for 5 d in various conditions (above plots (**a**) and below horizontal axis (**b**)). (**a**) Cells stained for B220 and IgA (isotype controls, **Supplementary Fig. 2**). Numbers in quadrants indicate percent B220$^+$IgA$^+$ cells (top right) or B220$^-$IgA$^+$ cells (bottom right). Data are representative of three independent experiments. (**b**) Concentration of IgA in coculture supernatants. *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**c**) Expression of *Aicda* mRNA (encoding activation-induced cytidine deaminase) in IgM$^+$IgD$^+$ cells cultured together with wild-type or *Tlr5*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs with or without flagellin. Data are representative of three independent experiments. (**d**) Immunohistochemistry of IgA$^+$ cells (green) in the small intestine ($n = 4$ mice per group). Scale bars, 50 μm. Data are representative of three independent experiments. (**e**) Flow cytometry of LPCs stained for B220 and IgA. Numbers above outlined areas indicate percent B220$^-$IgA$^+$ cells. Data are representative of three independent experiments.

most abundant immunoglobulin in the gut[20]. Intestinal IgA$^+$ plasma cells are generated mainly in GALT, including Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes, by a mechanism dependent on antigen, T cells and the formation of germinal centers[21–23]. Differentiated IgA$^+$ cells are 'imprinted' by GALT DC–derived retinoic acid for gut homing through the selective expression of gut-homing receptors, including integrin $\alpha_4\beta_7$ and CCR9 (ref. 7). However, reports have shown that IgA$^+$ cell development does not necessarily require T cell help and the formation of germinal centers[21,24] and that GALT DC–derived retinoic acid can potently act in synergy with cytokines produced by DCs and/or other cells to generate T cell–independent IgA$^+$ cells[7]. Furthermore, it seems that some IgM$^+$ B cells, especially peritoneal B1 cells, migrate directly to the gut lamina propria by a mechanism dependent on sphingosine 1-phosphate[25,26] and differentiate into IgA$^+$ plasma cells in the lamina propria with the help of stroma cells[24]. Commensal bacteria induce natural secretory IgA, and this process is mediated by DCs loaded with commensal bacteria[6,27]. Nevertheless, although published work has suggested the involvement of DCs in gut IgA production[28,29], it is unknown what subset of DCs is responsible for this event and how this is achieved. We thus examined whether CD11c^{hi}CD11b^{hi} LPDCs are involved in the generation of IgA$^+$ cells; we used SPDCs (TLR5$^-$TLR4$^+$) for comparison[18]. Flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs but not LPS-stimulated SPDCs efficiently induced the differentiation of B220$^-$ IgA$^+$ plasma cells in the absence of T cells in a TLR5-dependent way (**Fig. 3a,b** and **Supplementary Fig. 2** online). Expression of

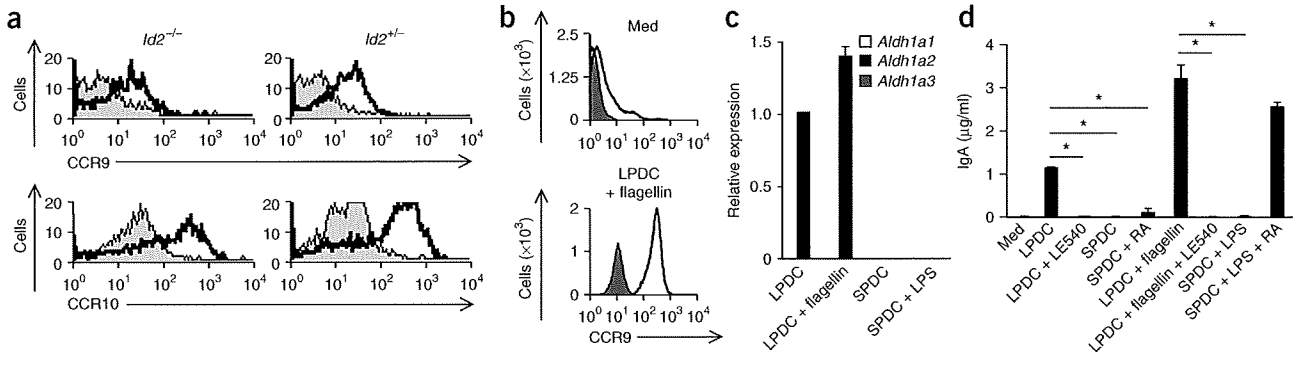

Figure 4 Function of retinoic acid released by CD11c^{hi}CD11b^{hi} LPDCs in IgA synthesis. (**a**) Flow cytometry of LPCs stained for B220, IgA, CCR9 or CCR10 (open histograms), gated on B220$^-$IgA$^+$ cells. Filled histograms, isotype control. Data are representative of three independent experiments. (**b**) Flow cytometry of peritoneal B220$^+$ cells cultured for 5 d with or without flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs. Data for CCR9 (open histograms) were acquired after gating on B220$^+$ cells (top) or B220$^-$IgA$^+$ cells (bottom). Filled histograms, isotype control. Data are representative of three independent experiments. (**c**) Quantitative real-time PCR of mRNA encoding retinal dehydrogenase isozymes (key) in CD11c^{hi}CD11b^{hi} LPDCs and SPDCs left unstimulated or stimulated with LPS or flagellin (horizontal axis). Data are representative of three independent experiments (mean and s.d.). (**d**) ELISA of IgA in supernatants of peritoneal B220$^+$ cells cultured for 5 d in various conditions (horizontal axis) with or without LE540 (1 μM) or retinoic acid (RA; 1 nM). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments.

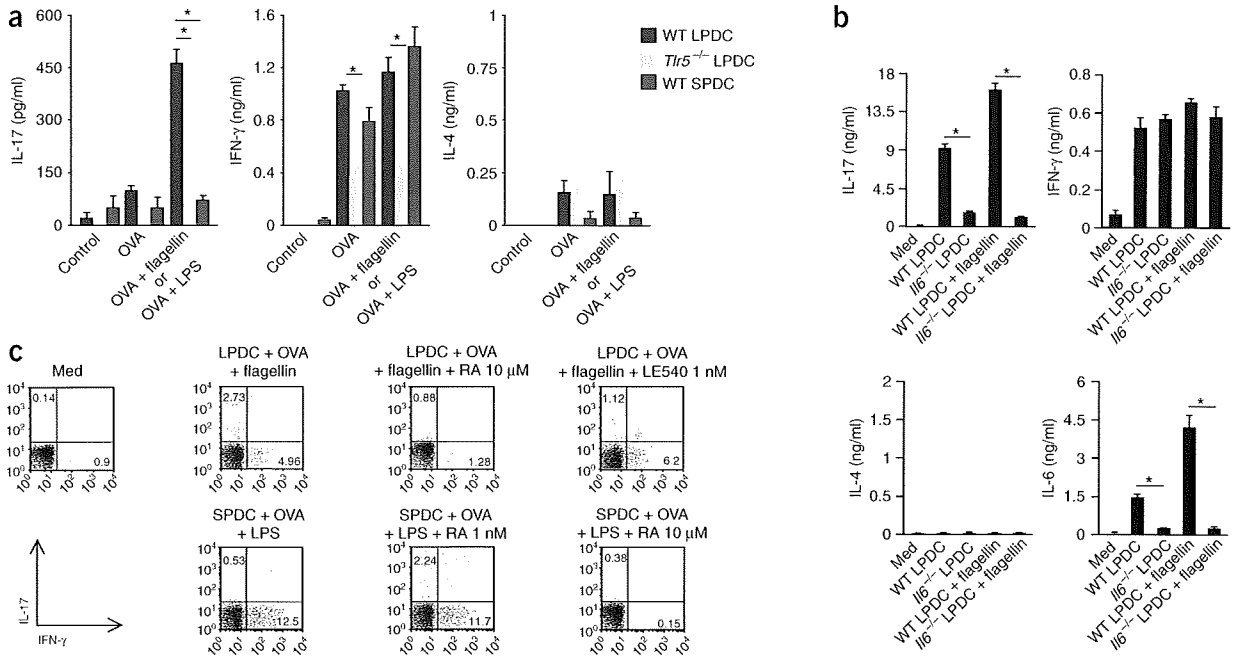

Figure 5 TLR5-dependent T_H-17 cell differentiation by CD11c^{hi}CD11b^{hi} LPDCs. (**a**) ELISA of IFN-γ, IL-17 and IL-4 in culture supernatants. CD11c^{hi}CD11b^{hi} LPDCs or SPDCs cultured for 12 h with OVA protein (100 μg/ml) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml) were injected on days 0 and 14 into the peritoneal cavities of naive *Tlr5*$^{-/-}$ mice (wild-type CD11c^{hi}CD11b^{hi} LPDCs) or *Tlr4*$^{-/-}$ mice (wild-type SPDCs) at a dose of 5 × 10^4 antigen-loaded cells per mouse; control mice were treated with PBS. At 1 week after the final immunization, splenocytes were collected and were cultured for 4 d with OVA protein (10 μg/ml) or with OVA peptide (amino acids 323–339; 10 μg/ml; **Supplementary Fig. 6**). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**b**) ELISA of cytokines in supernatants of OT-II transgenic CD4$^+$ T cells cultured for 4 d together with wild-type or *Il6*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs (conditions, horizontal axes). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**c**) Flow cytometry of OT-II transgenic CD4$^+$ T cells cultured for 4 d in various conditions (above plots) and stained intracellularly for IL-17 and IFN-γ (isotype controls, **Supplementary Fig. 7a**). Numbers in quadrants indicate percent IL-17$^+$IFN-γ$^-$ cells (top left) or IL-17$^-$IFN-γ$^+$ cells (bottom right). Data are representative of three independent experiments.

mRNA encoding activation-induced cytidine deaminase[30], an enzyme essential for class-switch recombination, was upregulated in naive B cells cultured together with flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs (**Fig. 3c**).

Although the results presented above demonstrated that CD11c^{hi}CD11b^{hi} LPDCs were able to induce T cell–independent differentiation of IgA$^+$ cells *in vitro*, we also examined the *in vivo* function of TLR5 in IgA synthesis by using GALT-deficient mice that intrinsically lack secondary lymphoid organs but have LPDCs. Mice lacking the transcription factors Id2 or RORγt, as well as bone marrow–reconstituted mice lacking lymphotoxin-α or both lymphotoxin-α and tumor necrosis factor, do not develop GALT, yet they retain intestinal IgA production[21,31]. Indeed, we detected many IgA$^+$ cells in the lamina propria of *Id2*$^{-/-}$ mice, which confirmed that gut IgA can be generated without GALT (**Fig. 3d,e**). Furthermore, we found no defects in the *in vitro* differentiation of IgA$^+$ plasma cells induced by CD11c^{hi}CD11b^{hi} LPDCs from peritoneal B cells isolated from *Id2*$^{-/-}$ mice (**Supplementary Fig. 3** online). Although *Tlr5*$^{-/-}$ mice did not have fewer IgA$^+$ B cells, *Id2*$^{-/-}$*Tlr5*$^{-/-}$ mice had far fewer IgA$^+$ cells in the lamina propria (**Fig. 3d,e**). Thus, TLR5 signaling in CD11c^{hi}CD11b^{hi} LPDCs is critical for GALT-independent IgA synthesis *in vivo*.

Retinoic acid in LPDC-induced IgA synthesis

We next examined the expression of gut-homing receptors on lamina propria IgA$^+$ cells in *Id2*$^{-/-}$ mice. Unexpectedly, B220$^-$IgA$^+$ plasma cells in the lamina propria of *Id2*$^{-/-}$ mice had high expression of CCR9, despite the lack of GALT in these mice (**Fig. 4a**). These cells also expressed CCR10, another chemokine receptor important for gut tropism[32]. As we did not detect high CCR9 expression on either peritoneal or splenic unstimulated B220$^+$ cells from wild-type or *Id2*$^{-/-}$ mice (**Fig. 4b** and data not shown), CCR9 might be induced on B cells only after their migration to the lamina propria in *Id2*$^{-/-}$ mice. In contrast, coculture with flagellin-treated CD11c^{hi}CD11b^{hi} LPDCs induced CCR9 expression on peritoneal B220$^-$IgA$^+$ cells (**Fig. 4b**). We therefore determined whether CD11c^{hi}CD11b^{hi} LPDCs synthesize retinoic acid, a mediator able to induce CCR9 expression. Retinal is converted into retinoic acid by retinal dehydrogenase enzymes. Although we detected no mRNA molecules encoding retinal dehydrogenase isoforms in SPDCs, CD11c^{hi}CD11b^{hi} LPDCs specifically expressed *Aldh1a2* mRNA, which encodes retinal dehydrogenase 2 (**Fig. 4c**). To determine if the CD11c^{hi}CD11b^{hi} LPDC–mediated development of IgA$^+$ cells was controlled by retinoic acid, we added the retinoic acid receptor inhibitor LE540 during the *in vitro* coculture of B cells and CD11c^{hi}CD11b^{hi} LPDCs. LE540 abrogated IgA production by B cells cultured together with flagellin-activated CD11c^{hi}CD11b^{hi} LPDCs (**Fig. 4d**). Moreover, supplementation of LPS-activated SPDCs with retinoic acid increased IgA concentrations to an extent similar to that induced by flagellin-activated CD11c^{hi}CD11b^{hi} LPDCs. Thus, the characteristic ability to synthesize retinoic acid grants CD11c^{hi}CD11b^{hi} LPDCs the ability to generate T cell–independent IgA$^+$ cells.

LPDC-induced T_H-17 cell differentiation

We next assessed the ability of CD11c^{hi}CD11b^{hi} LPDCs to induce antigen-specific T helper cell differentiation of ovalubumin

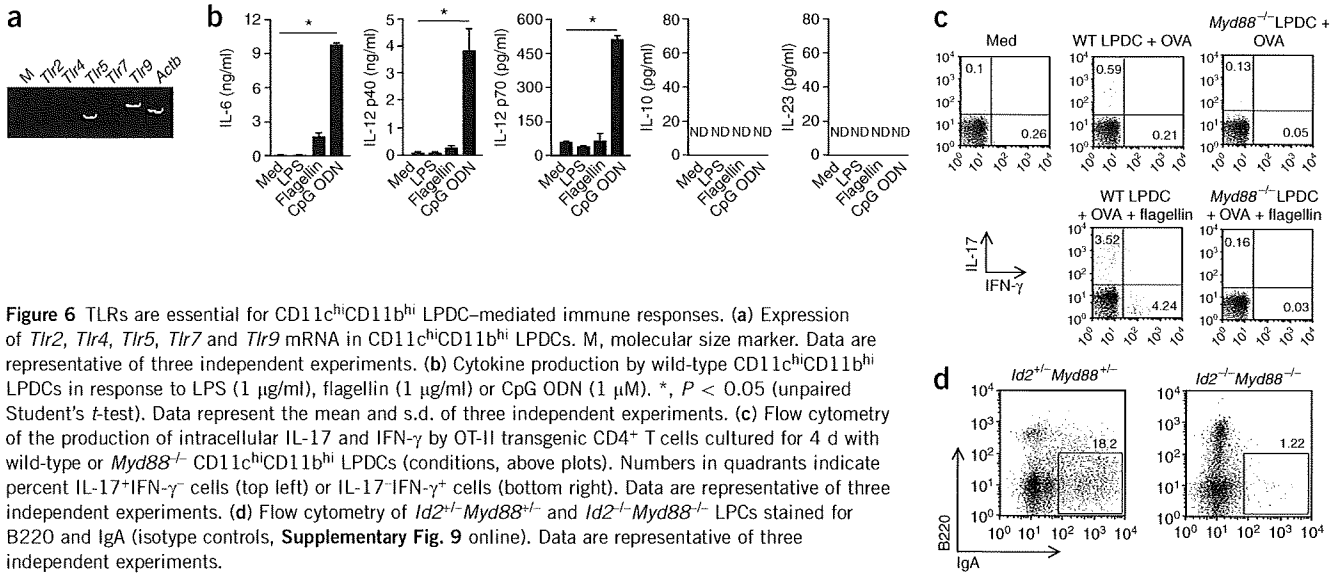

Figure 6 TLRs are essential for CD11c^{hi}CD11b^{hi} LPDC–mediated immune responses. (**a**) Expression of *Tlr2*, *Tlr4*, *Tlr5*, *Tlr7* and *Tlr9* mRNA in CD11c^{hi}CD11b^{hi} LPDCs. M, molecular size marker. Data are representative of three independent experiments. (**b**) Cytokine production by wild-type CD11c^{hi}CD11b^{hi} LPDCs in response to LPS (1 μg/ml), flagellin (1 μg/ml) or CpG ODN (1 μM). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**c**) Flow cytometry of the production of intracellular IL-17 and IFN-γ by OT-II transgenic CD4$^+$ T cells cultured for 4 d with wild-type or *Myd88*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs (conditions, above plots). Numbers in quadrants indicate percent IL-17$^+$IFN-γ$^-$ cells (top left) or IL-17$^-$IFN-γ$^+$ cells (bottom right). Data are representative of three independent experiments. (**d**) Flow cytometry of *Id2*$^{+/-}$*Myd88*$^{+/-}$ and *Id2*$^{-/-}$*Myd88*$^{-/-}$ LPCs stained for B220 and IgA (isotype controls, **Supplementary Fig. 9** online). Data are representative of three independent experiments.

(OVA)-specific OT-II-transgenic CD4$^+$ T cells. Although we detected only interferon-γ (IFN-γ)-producing cells in cocultures of OT-II T cells and LPS-stimulated SPDCs, we detected both IL-17- and IFN-γ-producing cells in cocultures of OT-II T cells and CD11c^{hi}CD11b^{hi} LPDCs; the numbers of IL-17- and IFN-γ-producing OT-II cells were further increased by flagellin stimulation of LPDCs[33–36] (**Supplementary Fig. 4a,b** online). In support of the idea that TLR5$^+$ CD11c^{hi}CD11b^{hi} LPDCs induce T_H-17 differentiation, naive CD4$^+$ T cells cultured together with wild-type CD11c^{hi}CD11b^{hi} LPDCs had higher expression of RORγt and IL-21, but those cultured together with *Tlr5*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs did not (**Supplementary Fig. 4c,d**). In contrast, other LPDC subsets (R1, R3 and R4) induced neither IL-17 nor IFN-γ production in response to flagellin (**Supplementary Fig. 5** online).

Next we examined *in vivo* the T helper cell responses of mice immunized with antigen-loaded DCs. We detected antigen-specific IFN-γ production after injection of both SPDCs and CD11c^{hi}CD11b^{hi} LPDCs, and this production was augmented by stimulation of TLR5 and TLR4 (**Fig. 5a** and **Supplementary Fig. 6** online). In addition, large amounts of IL-17 were produced by splenocytes from mice injected with flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs but not those injected with LPS-stimulated SPDCs. Those responses were impaired when mice were injected with *Tlr5*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs. As IL-6 is an essential cytokine for T_H-17 cell differentiation, and as CD11c^{hi}CD11b^{hi} LPDCs produced IL-6 in response to TLR5 stimulation (**Fig. 2b**), we then examined the involvement of IL-6 in CD11c^{hi}CD11b^{hi} LPDCs–induced T_H-17 cell differentiation[37]. Despite normal induction of IFN-γ, IL-17 production induced by flagellin-stimulated *Il6*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs was significantly lower than that elicited by flagellin-stimulated wild-type CD11c^{hi}CD11b^{hi} LPDCs (**Fig. 5b**).

A series of studies has shown that retinoic acid negatively regulates T_H-17 cell differentiation[38–40]. In agreement with those results, supplementation of cocultures of T cells and CD11c^{hi}CD11b^{hi} LPDCs with 10 μM retinoic acid effectively inhibited *in vitro* T_H-17 cell differentiation; retinoic acid supplementation also suppressed T_H1 cell differentiation (**Fig. 5c** and **Supplementary Fig. 7** online). However, we suspected that this concentration of retinoic acid may have been too high, as plasma retinoic acid concentrations are usually on the order of 10 nM and retinoic acid efficiently enhances the expression of gut-homing receptors on CD8$^+$ T cells even at a concentration of 0.1 nM (ref. 41). Notably, the retinoic acid inhibitor LE540 inhibited the differentiation of T_H-17 cells but not T_H1 cells, which suggested that retinoic acid from CD11c^{hi}CD11b^{hi} LPDCs is actually necessary for T_H-17 cell differentiation. In line with that observation, LPS-stimulated SPDCs induced T_H-17 cell differentiation to the same extent as flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs when cultured together with 1 nM retinoic acid, and 10 μM retinoic acid abolished T_H1 cell differentiation induced by LPS-stimulated SPDCs (**Fig. 5c** and **Supplementary Fig. 7**). Thus, retinoic acid at a low concentration acts as a positive regulator of T_H-17 cell differentiation, and the effect of retinoic acid on T_H-17 cell differentiation depends on its concentration.

CD11c^{hi}CD11b^{hi} LPDCs induce antigen-specific T_H-17 cells and T_H1 cells, but it is not clear whether these adaptive immune responses are protective against bacterial infection. T_H-17 cells constitute approximately 2% of the total CD4$^+$ T cell population in the small intestinal lamina propria of C57BL/6 mice without infection, and the number of T_H-17 cells did not change during the acute phase of oral *S. typhimurium* infection (**Supplementary Fig. 8a** online). Mice immunized with OVA-loaded CD11c^{hi}CD11b^{hi} LPDCs had greater proportions of T_H-17 and T_H1 cells in the lamina propria (**Supplementary Fig. 8b**). Challenge of the immunized mice with oral OVA further increased the proportions of lamina propria T_H-17 and T_H1 cells. Similarly, immunization with *S. typhimurium* flagellin–loaded wild-type LPDCs resulted in a significant increase in the proportion of lamina propria T_H-17 cells after oral challenge with *S. typhimurium*, ($P < 0.05$; **Supplementary Fig. 8c**) and resulted in partial protection against lethal challenge with *S. typhimurium* (**Supplementary Fig. 8d**), but similar immunization with *Tlr5*$^{-/-}$ LPDCs did not. Thus, CD11c^{hi}CD11b^{hi} LPDC–mediated immunization contributed to host defense against *S. typhimurium*.

TLR signals in LPDC-mediated inflammation

Although we demonstrated the importance of TLR5 in the activation of adaptive immunity by CD11c^{hi}CD11b^{hi} LPDCs, *Tlr5*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs nevertheless induced small amounts of IL-17- and IFN-γ-producing cells (**Supplementary Fig. 4a**). In

addition, we detected residual B220$^-$IgA$^+$ plasma cells in the lamina propria of *Id2*$^{-/-}$*Tlr5*$^{-/-}$ mice (**Fig. 3e**). Thus, other TLRs may contribute to such responses. Accordingly, CD11c^{hi}CD11b^{hi} LPDCs expressed TLR9 as well as TLR5 and produced proinflammatory cytokines in response to the TLR9 ligand CpG DNA (**Fig. 6a,b**). Notably, unlike wild-type CD11c^{hi}CD11b^{hi} LPDCs, *Myd88*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs failed to induce the *in vitro* differentiation of T_H-17 and T_H1 cells (**Fig. 6c**). Furthermore, B220$^-$IgA$^+$ cells were almost completely absent from the lamina propria of *Id2*$^{-/-}$*Myd88*$^{-/-}$ mice (**Fig. 6d**). These data collectively suggest that TLR signals in general are critical for CD11c^{hi}CD11b^{hi} LPDC–mediated activation of acquired immunity.

DISCUSSION

In this work we have demonstrated the unique characteristics of CD11c^{hi}CD11b^{hi} TLR5-expressing LPDCs. It is noteworthy that TLR5 activation by flagellin triggered CD11c^{hi}CD11b^{hi} LPDC–mediated adaptive immune responses. Studies have shown that adjuvant effects are associated with the induction of protective immunity in the intestine. Injection of the ligand for the receptor tyrosine kinase Flt3, which expands DC populations in the intestine, enhances both tolerance and immunity to orally administered antigens[42,43]. Relative to mice fed antigen alone, those receiving Flt3 ligand and antigen show greater susceptibility to the induction of oral tolerance[42]. However, such oral tolerance is abrogated and immune responses are induced when mice are fed the same antigen with an adjuvant such as IL-1 or cholera toxin[43]. Such findings indicate that DC activation is a crucial parameter determining whether tolerance or protective immunity is induced in the intestine. In physiological conditions, antigens such as food proteins may be presented by quiescent CD11c^{hi}CD11b^{hi} LPDCs in the absence of inflammation, leading to tolerance. However, when inflammatory stimuli such as flagellin are present, CD11c^{hi}CD11b^{hi} LPDCs will undergo maturation, release inflammatory cytokines and initiate protective acquired immunity.

Commensal bacteria are present at a high density in the intestinal lumen (up to 1×10^{12} bacteria per gram of lumenal contents). Most commensal organisms reside outside the layer of mucus that covers the intestinal epithelial cells. Some bacteria penetrate the enterocyte epithelial layer but are rapidly killed by macrophages[44]. However, some commensal bacteria are ingested by DCs, where they survive for several days[6]. Moreover, intraepithelial DCs send protrusions into the lumen of the small intestine in a CX3CR1-dependent way and directly sample lumenal commensal bacteria[45,46]. Commensal bacteria–loaded DCs mediate the induction of natural secretary IgA[27], and germ-free mice have a profound deficiency in IgA production in the intestinal mucosa[44]. Thus, the presence of intestinal microbiota influences IgA production in the intestine. As the induction of B220$^-$IgA$^+$ plasma cells was impaired in *Id2*$^{-/-}$*Tlr5*$^{-/-}$ mice and was almost completely abrogated in *Id2*$^{-/-}$*Myd88*$^{-/-}$ mice, GALT-independent IgA production seems to be mediated by TLR stimulation. These results indicate that TLRs represent a 'missing link' between commensal bacteria and IgA synthesis in the lamina propria.

The ability to synthesize retinoic acid enables CD11c^{hi}CD11b^{hi} LPDCs to modulate various immune response parameters. CD11c^{hi}CD11b^{hi} LPDCs induced IgA$^+$ cell differentiation without T cell help in a retinoic acid–dependent way. In the process, CD11c^{hi}CD11b^{hi} LPDCs also promoted the upregulation of CCR9 expression on B cells. Notably, IgA$^+$ plasma cells had high expression CCR9 in the lamina propria of GALT-deficient *Id2*$^{-/-}$ mice. These results were unexpected, because GALT DCs are believed to 'imprint' gut tropism on lymphocytes. The ability of CD11c^{hi}CD11b^{hi} LPDCs to induce CCR9 on differentiated IgA$^+$ plasma cells may promote retention in the lamina propria, as the CCR9 ligand CCL25 is abundantly secreted by the crypt epithelium[21]. Although previous studies have shown that retinoic acid negatively regulates T_H-17 cell differentiation, here we have shown that the effect of retinoic acid on T helper cell differentiation depended strictly on its concentration. It is difficult to determine the local concentrations of retinoic acid secreted by CD11c^{hi}CD11b^{hi} LPDCs. However, studies intensively examining the concentration of retinoic acid secreted by GALT DCs in the work of T cell 'imprinting' have shown that 1 nM of retinoic acid is the optimum concentration for the induction of gut-homing receptors on T cells[41]. Notably, high concentrations of retinoic acid inhibited the differentiation of both T_H1 and T_H-17 cells, which suggested that the inhibitory effect of high concentrations of retinoic acid is not specific for T_H-17 polarization. Such observations indicate that the effect of retinoic acid on T_H-17 cell differentiation should be considered more cautiously. In any case, like the CD11c^{hi}CD11b^{hi} LPDC–induced differentiation of IgA$^+$ plasma cells, T_H-17 cell differentiation required retinoic acid. Unlike other conventional DCs, LPDCs can induce the differentiation of antigen-specific T_H-17 cells as well as T_H1 cells in response to TLR stimulation. The ability to produce retinoic acid may support this unique function of CD11c^{hi}CD11b^{hi} LPDCs.

We conclude that CD11c^{hi}CD11b^{hi} LPDCs may work against bacterial infection by inducing 'local' IgA secretion and 'systemic' T helper cell responses through TLR stimulation. As IL-17 can influence cytokine production by a wide range of cell types and can induce the activation and migration of neutrophils[47], CD11c^{hi}CD11b^{hi} LPDCs and T_H-17 cells may modulate the pathogenesis of intestinal bowel diseases such as Crohn's disease. In addition, the ability of CD11c^{hi}CD11b^{hi} LPDCs to induce the differentiation of T_H1 and IgA$^+$ cells suggests that CD11c^{hi}CD11b^{hi} LPDCs might be useful targets of mucosal vaccination.

METHODS

Mice. *Tlr4*$^{-/-}$ (C57BL/6) mice, *Tlr5*$^{-/-}$ mice (C57BL/6), *Id2*$^{-/-}$ mice and *Myd88*$^{-/-}$ mice have been described[18,48]. *Il6*$^{-/-}$ mice (C57BL/6) and OT-II-transgenic mice (C57BL/6) were provided by M. Kopf[49] and W.R. Heath[50], respectively. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases at Osaka University.

Reagents. LPS, flagellin and CpG oligodeoxynucleotides (ODN 1668) were purified as described[18]. *S. typhimurium* flagellin was from Invivogen. All-*trans* retinoic acid (Sigma) was dissolved in dimethyl sulfoxide, was stored at –80 °C with light interception and was added to cultures at a final concentration of 1 nM. LE540 (Wako) was dissolved in dimethyl sulfoxide and was added to cultures at a final concentration of 1 μM.

Cells. Segments of the small intestine were treated for 30 min at 37 °C with PBS containing 10% (vol/vol) FCS, HEPES (20 mM), pH 7.4, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), EDTA (10 mM) and polymyxin B (10 μg/ml; Calbiochem) for removal of epithelial cells, then were washed extensively with PBS. Segments of the small intestine and spleen were digested for 45–90 min with continuous stirring at 37 °C with collagenase D (400 Mandl units/ml; Roche) and DNase I (10 μg/ml; Roche) in RPMI 1640 medium plus 10% (vol/vol) FCS. EDTA was added (final concentration, 10 mM) and cell suspensions were incubated for an additional 5 min at 37 °C. Cells were spun through a 17.5% (wt/vol) solution of Accudenz (Accurate Chemical & Scientific) for enrichment for DCs. The cells obtained were incubated with fluorescein isothiocyanate–conjugated antibody to CD11b (anti-CD11b; M170; 557396) and phycoerythrin-conjugated anti-CD11c (HL3; 557401; both from BD Pharmingen) after blockade of Fc receptors. DC subsets were sorted on the basis of their expression of CD11c and CD11b with a FACSVantage SE or FACSAria (BD Biosciences). The purity of the sorted DCs

was routinely over 95%. For morphological studies, cytospin preparations from purified DC subsets were stained with May-Grunwald-Giemsa solution. For analysis of leukocytes, cells were subjected to density-gradient centrifugation in 40% to 75% (vol/vol) Percoll (approximately density, 1.058 g/ml and 1.093 g/ml, respectively) after enzyme treatment. Cells collected from the interface were washed and were used as lamina propria leukocytes in assays. Naive $CD4^+$ T cells from the spleens of OT-II transgenic mice and $B220^+$ cells from the peritoneal cavities of C57BL/6 mice were purified by magnetic sorting with mouse anti-CD4 beads and mouse anti-B220 beads, respectively. Peritoneal cells from C57BL/6 mice were incubated with fluorescein isothiocyanate–conjugated conjugated anti-IgD (11-26c.2a; 553439; BD Pharmingen) and phycoerythrin-indotricarbocyanine–conjugated anti-IgM (R6-60.2; 553409; BD Pharmingen) after blockade of Fc receptors. Naive B cells were sorted on the basis of their expression of IgD and IgM with a FACSVantage SEM or FACSAria (BD Biosciences). The purity of the sorted cells was routinely over 95%.

***In vitro* T cell differentiation.** OT-II transgenic $CD4^+$ T cells (1×10^6) were cultured with $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs (1×10^5) in the presence of OVA protein (100 μg/ml), unsupplemented or supplemented with flagellin (1μg/ml) or LPS (1 μg/ml), respectively. After 4 d, cells were restimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Calbiochem) in the presence of GolgiStop (BD Pharmingen), then cells producing IL-17 and IFN-γ were analyzed by flow cytometry.

Immunization. $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs were cultured for 12 h with OVA protein (100 μg/ml) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml). Antigen-loading cells (5×10^4 per mouse) were injected on days 0 and 14 into the peritoneal cavities of naive *Tlr5*$^{-/-}$ mice ($CD11c^{hi}CD11b^{hi}$ LPDCs) or *Tlr4*$^{-/-}$ mice (SPDCs); control mice were treated with PBS. At 1 week after the final immunization, splenocytes were collected and were cultured for 4 d with OVA protein (10 μg/ml). The concentration of IFN-γ, IL-17 and IL-4 in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA).

***In vitro* IgA^+ plasma cell differentiation.** Peritoneal IgM^+IgD^+ cells or $B220^+$ cells (1×10^6) were cultured in medium supplemented with B cell–activating factor (50 ng/ml) together with $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs (1×10^5 to 5×10^5) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml), respectively. After 5 d, cells were analyzed by flow cytometry and the concentration of IgA in culture supernatants was measured by ELISA.

Flow cytometry. Before staining, Fc receptors were blocked for 15 min at 4 °C. Low-density LPCs were stained with the following biotinylated monoclonal antibodies: anti-CD11b (M1/70; 557395), anti-CD11c (HL3; 553800), anti-CD40 (3/23; 553789), anti-CD80 (16-10A1; 553767), anti-CD86 (GL1; 553690), anti-I-A/I-E (2G9; 553622) and anti-CD103 (M290; 557493; all from BD Pharmingen); anti-F4/80 (A3-1; MF48015; Caltag Laboratories); and anti-DEC-205 (NLDC-145; CL89145PE; Cedarlane Laboratories). The surfaces of cocultured T cells were stained with fluorescein isothiocyanate–labeled anti-CD4 (L3T4; 553055; BD Pharmingen). Then, cells were fixed and made permeable with Cytofix/Cytoperm (BD Pharmingen) and were stained intracellularly with phycoerythrin-labeled anti-IL-17 (TCC11-18H10.1; 559502) and allophycocyanin-labeled anti-IFN-γ (XMG1.2; 554413; both from BD Pharmingen). The surfaces of cocultured B cells or lamina propria leukocytes were stained with phycoerythrin-labeled anti-B220 (RA3-6B2; 553090; BD Pharmingen). Then, cells were fixed and made permeable with Cytofix/Cytoperm and were stained intracellularly with fluorescein isothiocyanate–labeled anti-IgA (C10-3; 559354) or were incubated with biotin-conjugated IgA (C10-1; 556978) and then stained intracellulary with allophycocyanin-labeled streptavidin (all from BD Pharmingen). CCR9 expression on lamina propria leukocytes and cocultured B cells was assessed with rat anti–mouse CCR9 (242503; FAB2160A; R&D Systems). CCR10 expression on lamina propria leukocytes and cocultured B cells was assessed with rat anti–mouse CCR10 (248918; FAB2815A; R&D Systems). Samples were acquired on a FACSCalibur with CELLQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

RT-PCR and quantitative real-time PCR. RNA (1 μg) was reverse-transcribed with Superscript2 (Invitrogen) according to the manufacturer's instructions with random hexamers as primers. Primer pairs specific for *Tlr2*, *Tlr4*, *Tlr5*, *Tlr7*, *Tlr9*, *Aicda*[30] or *Actb* (**Supplementary Table 1** online) and Taq polymerase[18] (Takara Shuzo) were used for PCR of 25 cycles at 97 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; products were separated by agarose gel electrophoresis. A 7700 Sequence Detector (Applied Biosystems) was used for quantitative real-time PCR of cDNA amplified as described above with 2× PCR Master Mix (Applied Biosystems) and primers for 18S rRNA (as an internal control; Applied Biosystems) or primers specific for *Tlr5*, *Rorc*, *Aldh1a1*, *Aldh1a2* or *Aldh1a3* (Applied Biosystems), in a final volume of 25 μl. After incubation at 95 °C for 10 min, products were amplified by 35 cycles of 95 °C for 15 s, 60 °C for 60 s and 50 °C for 120 s.

Measurement of cytokines in supernatants. The concentrations of IFN-γ, IL-17, IL-4, IL-6, IL-10 and IL-12p40 were measured with the Bio-plex system (Bio-Rad) according to the manufacturer's instructions. The concentrations of IL-21, IL-23 and IgA were determined by ELISA (R&D Systems, eBioscience and Southern Biotech, respectively).

Immunohistochemical analysis. For analysis of the number of IgA^+ cells in the small intestinal lamina propria, fluorescein isothiocyanate–conjugated anti–mouse IgA (C10-3; 559354; BD Pharmingen) was applied overnight at 4 °C to sections cut from frozen tissue. Immunohistochemical staining was analyzed with a Radiance 2100 Bio-Rad confocal laser microscope (Bio-Rad).

Bacterial infection. *S. enterica* serovar *typhimurium* has been described[18]. *S. typhimurium* was grown in Luria-Bertani medium without shaking at 37 °C. The concentration of bacteria was determined on the basis of the absorbance at 600 nm. Bacteria were injected orally into mice.

Statistical analysis. Statistical significance was evaluated with an unpaired two-tailed Student's *t*-test in all experiments except **Supplementary Figure 8d**. A P value of less than 0.05 was considered significant. Kaplan-Meier plots and log-rank tests were used to assess the survival differences of control and mutant mice after bacterial infection (**Supplementary Fig. 8d**).

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org/): A002297 and A001174.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.F. and S.U. did most of the experiments; S.U., K.J.I. and M.H.J. designed all the experiments; B.-G.Y. helped with the immunohistochemical analysis; Y.-J.J. and M.N. helped to isolate cells; S.S., T.T. and M.Y. provided advice for the experiments; Y.Y. provided *Id2*$^{-/-}$ mice; H.K. and M.M. provided advice for the experiments and manuscript; S.U. and S.A. prepared the manuscript; and S.A. directed the research.

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