This is the first report of the expression of T_H17-related cytokines in the airway tissues in severe asthma. Although we did not perform colocalization studies, the pattern of the immunoreactive cells in the submucosa suggests that this new subset of T cells may be involved in the inflammatory process in severe asthma. IL-17 has been associated with the activation of epithelial cells *in vitro* and the induction of IL-6 and IL-8 with downstream effects on neutrophil recruitment and activation.[7] We and others have reported an upregulation of IL-8 in severe asthma.[8] Neutrophils were also shown to be increased in severe asthma by many groups,[9] and this phenomenon may be IL-17–driven. We have also previously reported that IL-17 is increased in chronic sinusitis and that its expression is resistant to steroids.[4] Steroid unresponsiveness in severe asthma has been attributed to the presence of neutrophilic inflammation and an upregulation of the glucocorticoid receptor β isoform. T_H17-related cytokines have been implicated in the pathogenesis of a number of diseases that do not respond well to corticosteroids. Recently McKinley et al[10] have shown in a murine model that T_H17 cells not only are proinflammatory cells but also may induce steroid resistance. It is possible that steroid hyporesponsiveness in subjects with severe asthma may also relate to the presence of IL-17A and IL-17F. IL-17 has also been reported to affect structural cells and to stimulate the production of profibrotic cytokines and extracellular matrix proteins. This feature of airway remodeling in severe asthma may be attributable to an excess of these cytokines. If so, targeting IL-17 cytokines may be of value in the therapy of severe asthma, in which steroid resistance, neutrophilic inflammation, and airway remodeling are substantial.

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Toll-like receptor 3 enhances late-phase reaction of experimental allergic conjunctivitis

To the Editor:

Toll-like receptors (TLRs) are well-known key receptors of the innate immune system. *TLR3* recognizes double-stranded RNA, a component of the lifecycle of most viruses, mimicking polyinosinic:polycytidylic acid (polyI:C). Although a relationship between viral infection and allergic inflammation has been reported, the function of *TLR3* in allergic inflammation remains to be defined. Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions; the degree of eosinophil infiltration in the conjunctiva reflects the degree of its late-phase reaction.[1,2] Using our model of murine experimental allergic conjunctivitis (EAC)[1] and *TLR3* knockout (KO) and *TLR3* transgenic (Tg) mice (*TLR3*KO and *TLR3*Tg mice, respectively), we assessed directly the role of *TLR3* in conjunctival eosinophil infiltration.

BALB/c mice purchased from CLEA (Tokyo, Japan) were sensitized at 6 to 12 weeks of age. *TLR3*KO and *TLR3*Tg mice were generated as previously described,[3,4] back-crossed more than 7 generations to BALB/c mice, and subjected to EAC at 9 to 15 weeks of age. Age-matched wild-type BALB/c mice were used as control animals. The experiments were conducted with a protocol approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Short ragweed pollen (RW) was purchased from Polysciences, Inc (Warrington, Pa), and aluminum hydroxide (alum) was purchased from Sigma-Aldrich Corp (St Louis, Mi). The mice were immunized with an intracutaneous injection into the left hind footpad of RW adsorbed on alum (200 μg of RW and 2.6 mg of alum) on day 0. On day 7, they received an intraperitoneal injection of RW adsorbed on alum, and on day 18, their eyes were challenged with RW in PBS (500 μg in 5 μL per eye) or with PBS alone (5 μL per eye).[1] Their eyes, including the conjunctiva, were harvested 24 hours after the last challenge, fixed in 10% neutral buffered formalin, and embedded in paraffin blocks for histologic analysis. Vertical 6-μm-thick sections were mounted on microscope slides, deparaffinized, and stained with Luna stain,[1,2] which identifies erythrocytes and eosinophil granules. Using the entire section from the central portion of the eye, including the pupil and optic nerve head, we counted infiltrating eosinophils in the lamina propria mucosae of the tarsal conjunctiva. Cell counts were expressed as the number of infiltrating eosinophils per unit area (0.1 mm^2) measured with image software (Scion Corp, Frederick, Md).[1,2] Quantitative RT-PCR

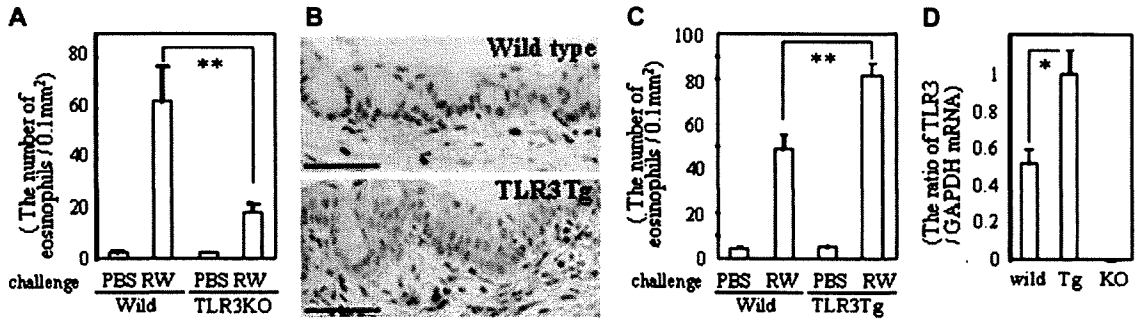

FIG 1. A, Eosinophil infiltration in *TLR3*KO mice. B, Eosinophil infiltration into the conjunctiva of ragweed-challenged wild-type and *TLR3*Tg mice was detected with Luna's method. Scale bars = 50 μm. C, Eosinophil infiltration in *TLR3*Tg mice. D, *TLR3* mRNA expression in eyelids. Data are shown as the means ± SEMs of samples from 3 mice. $*P < .05$. In Fig 1, *A* and *C*, data are shown as the means ± SEMs of samples from all 12 mice examined in 3 groups of 4 mice each. $**P < .01$.

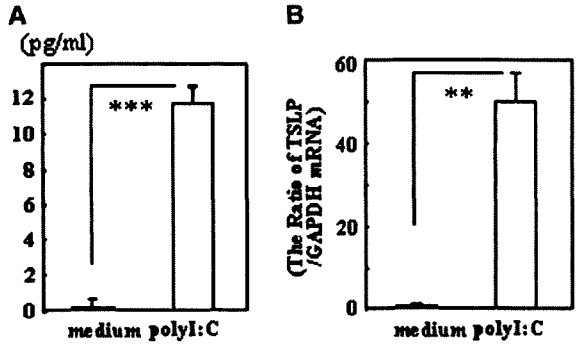

FIG 2. A, TSLP production. B, *TSLP* mRNA expression. The *y-axis* shows the increase in specific mRNA over that seen in medium samples. Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 μg/mL polyI:C and then incubated for 24 (Fig 2, *A*) or 6 (Fig 2, *B*) hours. The data are representative of 3 independent experiments and shown as the means ± SEMs of 4 samples. $***P < .0005$, $**P < .01$.

of *TLR3*-specific mRNA in the eyelids was performed as previously reported.[1,2] Briefly, the upper and lower lids were collected 6 hours after the last RW challenge and homogenized in liquid nitrogen. Total RNA was extracted with the RNeasy mini kit (Qiagen, Tokyo, Japan). ReverTraAce (TOYOBO, Otsu, Japan) was used for reverse transcription. The primers and probes for mouse *TLR3* and *glyceraldehyde-3-phosphate dehydrogenase* were from Applied Biosystems (Foster City, Calif). The results were analyzed with sequence detection software (Applied Biosystems). Data were expressed as the mean ± SE, and statistical analyses were performed by means of ANOVA or the Student *t* test, as appropriate.

First, we compared eosinophil infiltration in *TLR3*KO and wild-type mice. Although sensitization without challenge did not affect the number of eosinophils, after sensitization and challenge, their number in the lamina propria mucosae of the conjunctiva was significantly increased in both *TLR3*KO and wild-type mice; however, it was significantly lower in *TLR3*KO than in wild-type mice (Fig 1, *A*). Next we compared eosinophil infiltration in *TLR3*Tg mice and wild-type mice. The numbers of eosinophils in TLR3Tg mice after sensitization and challenge were significantly greater than in wild-type mice (Fig 1, *B* and *C*). Furthermore, we have confirmed that *TLR3* mRNA expression in the eyelids of *TLR3*Tg mice was greater than that of wild-type mice after sensitization with challenge and that *TLR3* mRNA expression in the eyelids of *TLR3*KO mice was undetectable (Fig 1, *D*). These results suggest that TLR3 positively regulates late-phase reaction of EAC, which causes reduced eosinophilic conjunctival inflammation in *TLR3*KO mice and increased it in *TLR3*Tg mice.

We also examined whether sensitization with RW induced RW-specific immune responses equally in wild-type, *TLR3*KO, and *TLR3*Tg mice. It produced an increase in IgE and IgGl antigen–specific antibody responses equally in all 3 groups of mice (data not shown), suggesting that their sensitization to RW was equivalent.

Our results showed that TLR3 could regulate allergic inflammation in the absence of an exogenous viral infection or TLR3 ligand. It is reported that in the absence of viral infection, TLR3 can amplify immune responses during acute inflammatory processes, which might involve stimulation of TLR3 by endogenous RNA from necrotic cells.[5] It is also possible that endogenous RNA from tissue or cells might stimulate TLR3 in our allergic conjunctivitis model. On the other hand, there is a report that a TLR3 ligand can suppress allergic inflammation.[6]

Although the function of TLR3 in allergy remains to be defined, the expression of thymic stromal lymphopoietin (TSLP), which plays a key role in allergic inflammation, is reportedly induced by stimulation with the TLR3 ligand in airway epithelial cells and keratinocytes.[7] TSLP is highly expressed by airway epithelial cells of asthmatic patients and keratinocytes in skin lesions of patients with atopic dermatitis. We previously reported that human ocular surface epithelium expressed TLR3[8,9] and that cytokine production was upregulated by polyI:C, a TLR3 ligand.[9] We also confirmed that TSLP is induced by means of stimulation with the TLR3 ligand polyI:C in human conjunctival epithelial cells (Fig 2 and see the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org). It is possible that TLR3 positively regulates the late-phase reaction of EAC through the induction of TSLP. Further investigations are required to identify the precise molecular mechanisms of allergic conjunctivitis in the murine model.

Elsewhere, we showed that EP3 is expressed in the ocular surface and that the prostaglandin E_2–EP3 pathway in

conjunctival epithelium works as a negative regulator for allergic conjunctivitis.[1] It is evident that ocular surface epithelial cells regulate the inflammation of allergic conjunctivitis. The actual role of TLR3 in conjunctival inflammation must be further investigated.

In summary, we demonstrated that TLR3 positively regulates late-phase reaction of EAC, which caused reduced eosinophilic conjunctival inflammation in *TLR3*KO mice and pronounced it in *TLR3*Tg mice.

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The carbohydrate galactose-α-1,3-galactose is a major IgE-binding epitope on cat IgA

To the Editor:

Cross-reactive carbohydrate determinants are widely occurring IgE epitopes. Glycan-related IgE reactivity has been demonstrated in most allergen sources, especially in the plant kingdom.[1] The clinical effect of these cross-reactive carbohydrate determinants is debated.

TABLE I. Comparison of monoclonal antigalactose reactivity to solid phase bound α-gal, cat IgA, and recombinant Fel d 1 (rFel d 1) by ELISA

Antigen	OD (450 nm)
α-gal	0.69
Cat IgA	0.67
rFel d 1	0.05

We were recently able to show that IgE Abs to the cat IgA, present in cat-sensitized patients, are mainly directed to a glycan moiety localized on the α-chain.[2] In addition, we have reported that these carbohydrates are present on IgM Abs from cat, as well as on IgM from many different mammalian species, but not human immunoglobulins.[3] Interestingly, IgE antibodies to cat IgM and cat IgA show a complete cross-reactivity, whereas cat IgG does not, suggesting an identical oligosaccharide on the 2 former immunoglobulin classes. Because this is the first mammalian carbohydrate IgE epitope found, it is of major interest to identify the carbohydrate structure responsible for the broad cross-reactivity.

Chung et al[4] have recently investigated subjects with anaphylactic reactions after treatment with the drug cetuximab, a chimeric mouse–human IgG_1 mAb against the epidermal growth factor receptor, which is approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck. The authors found that a carbohydrate epitope on the mouse Fab portion, galactose-α-1,3-galactose, a part of the Galα1,3Galß1,4GlcNAc-R (α-gal) epitope, was responsible for the IgE binding. Furthermore, in most subjects, the IgE antibodies against cetuximab were present in serum before therapy.

The α-gal epitope is expressed on many different glycoproteins in mammals, except for old world monkeys, apes, and human beings. Species lacking the α-gal residues produce large quantities of IgG antibodies to this epitope.[5] Studies have demonstrated that approximately 1% of antibodies in all healthy subjects are directed to α-gal.[6] These antibodies also react with closely related carbohydrate structures in the ABO blood group and are one of the major obstacles in xenotransplantation.

Here we investigated whether α-gal is present on cat IgA and whether it is a major epitope responsible for IgE binding to cat IgA.

Cat IgA was purified from cat serum,[3] and α-gal–human serum albumin was obtained from Dextra Laboratories, Reading, United Kingdom. To investigate the presence of α-gal on cat IgA, a monoclonal anti-Gal antibody was used in ELISA. Plates were coated with 5 μg/mL α-gal, cat IgA, or recombinant Fel d 1,[7] which was included as negative control. Incubation with monoclonal anti-Gal antibodies (Alexis Biochemicals, Lausen, Switzerland), diluted 1:25, was followed by antimouse–IgG-alkaline phosphatase (Dako, Glostrup, Denmark) and substrate solution (Sigma, Steinheim, Germany). We found that the anti-Gal reactivity to α-gal and cat IgA was almost identical, whereas no reactivity was detected to recombinant Fel d 1 (Table I).

Twenty sera from the United States, 9 from patients who were found to have IgE antibodies to the α-gal epitope on cetuximab by using the streptavidin CAP technique,[8] (range, 0.79 to >100 kilo

METHODS

Primary human conjunctival epithelial cells

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Declaration of Helsinki. The purposes of our research and the experimental protocol were explained to all patients, and their prior written informed consent was obtained.

For ELISA and real-time quantitative PCR, we harvested primary human conjunctival epithelial cells from conjunctival tissue obtained at the time of conjunctivochalasis surgery. Cells were cultured by using a modification of previously described methods.[E1] Briefly, conjunctival tissues were washed and immersed for 1 hour at 37°C in 1.2 U/mL purified Dispase (Roche Diagnostic Ltd, Basel, Switzerland). Epithelial cells were detached, collected, and cultured in low-calcium k-SFM medium supplemented with 0.2 ng/mL human recombinant epidermal growth factor (Invitrogen, Carlsbad, Calif), 25 mg/mL bovine pituitary extract (Invitrogen), and 1% antibiotic-antimycotic solution. Cell colonies usually became obvious within 3 or 4 days. After reaching 80% confluence in 7 to 10 days, the cells were seeded, and after reaching subconfluence, they were used in subsequent procedures.

ELISA

Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 μg/mL polyI:C and then incubated for 24 hours. The amount of TSLP proteins was determined by using ELISA. TSLP release into culture supernatants was quantitated by using the Human TSLP DuoSet (R&D Systems, Inc, Minneapolis, Minn), according to the manufacturer's instructions.

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems), according to previously described procedures.[E2] The initial amount of RNA used for reverse transcription to cDNA was approximately 1 μg. The cDNA was used at the original concentration for quantitative PCR. The primers and probes for human *TSLP* and human *glyceraldehyde-3-phosphate dehydrogenase* were from Perkin-Elmer Applied Biosystems. Quantitative PCR was used to measure the expression of *TSLP* mRNA in primary human conjunctival epithelial cells treated for 0, 1, 3, or 6 hours with 10 μg/mL polyI:C. The quantification data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

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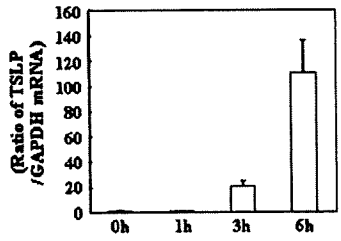

FIG E1. Increased *TSLP* mRNA expression by TLR3 stimulated with polyI:C. The *y-axis* shows the increase in specific mRNA over 0-hour samples or medium samples. The x-axis shows the time after stimulation. The data are presented as the means ± SEMs of 3 samples.

nature immunology

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

Satoshi Uematsu[1,2,12], Kosuke Fujimoto[1,2,12], Myoung Ho Jang[3], Bo-Gie Yang[1], Yun-Jae Jung[4], Mika Nishiyama[5], Shintaro Sato[6], Tohru Tsujimura[7], Masafumi Yamamoto[8], Yoshifumi Yokota[9], Hiroshi Kiyono[6], Masayuki Miyasaka[5], Ken J Ishii[1,10,11] & Shizuo Akira[1,2,10]

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.

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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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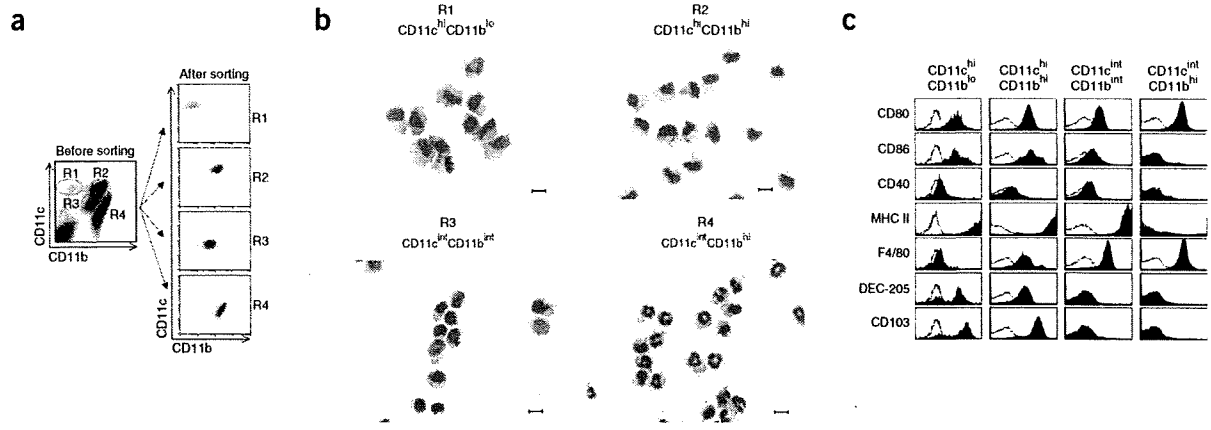

Figure 1 Four subsets of CD11c$^+$ LPCs in the small intestine. (**a**) Flow cytometry of intestinal low-density LPCs stained for CD11b and CD11c, before and after sorting. (**b**) May-Grunwald-Giemsa staining of four leukocyte subsets (gated in **a**) from the lamina propria. Scale bars, 10 μm. (**c**) 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.

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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

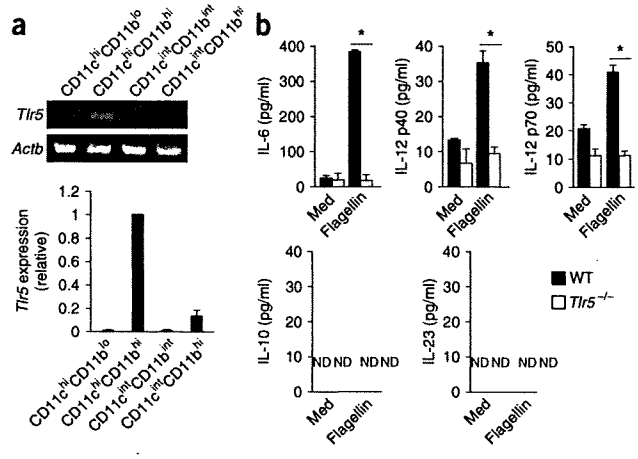

Figure 2 CD11c^{hi}CD11b^{hi} LPDCs specifically express TLR5. (**a**) RT-PCR (top) and quantitative real-time PCR (bottom) of *Tlr5* expression in the four leukocyte lamina propria subsets. *Actb* encodes β-actin (top, loading control). Expression (bottom) is relative to that of *Actb*. Data are representative 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.

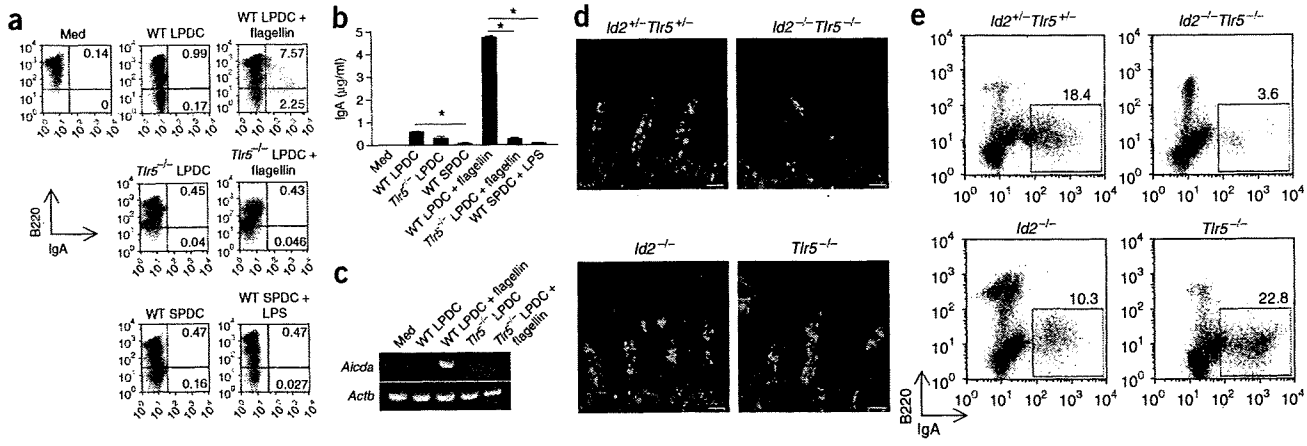

Figure 3 CD11c^{hi}CD11b^{hi} LPDCs induce IgA$^+$ plasma cell differentiation. (**a**,**b**) Flow cytometry (**a**) and ELISA (**b**) of peritoneal IgM$^+$IgD$^+$ cells cultured for 5 d in various conditions (above plots (**a**) and below horizontal axis (**b**)). (**a**) Cells stained for B220 and 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

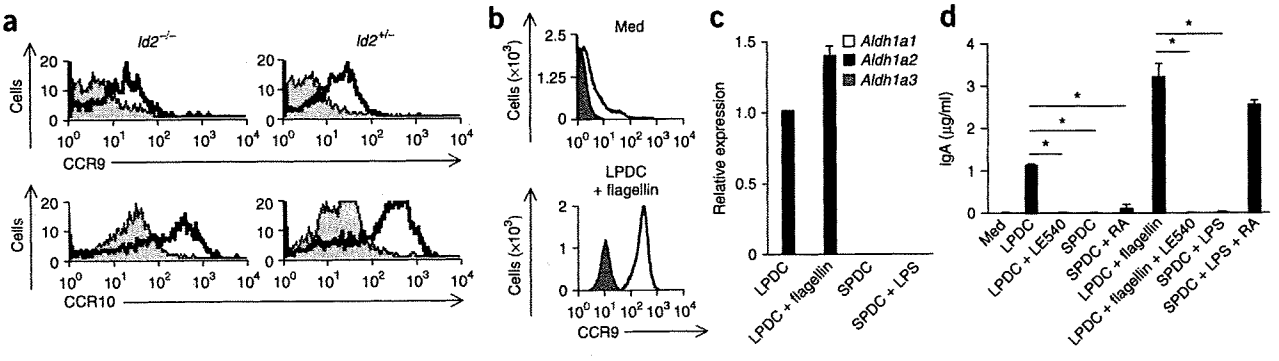

Figure 4 Function of retinoic acid released by CD11c^{hi}CD11b^{hi} LPDCs in IgA synthesis. (**a**) Flow cytometry of LPCs stained for B220, IgA, CCR9 or CCR10 (open histograms), gated on B220$^-$IgA$^+$ cells. Filled histograms, isotype control. Data are representative of three independent experiments. (**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.

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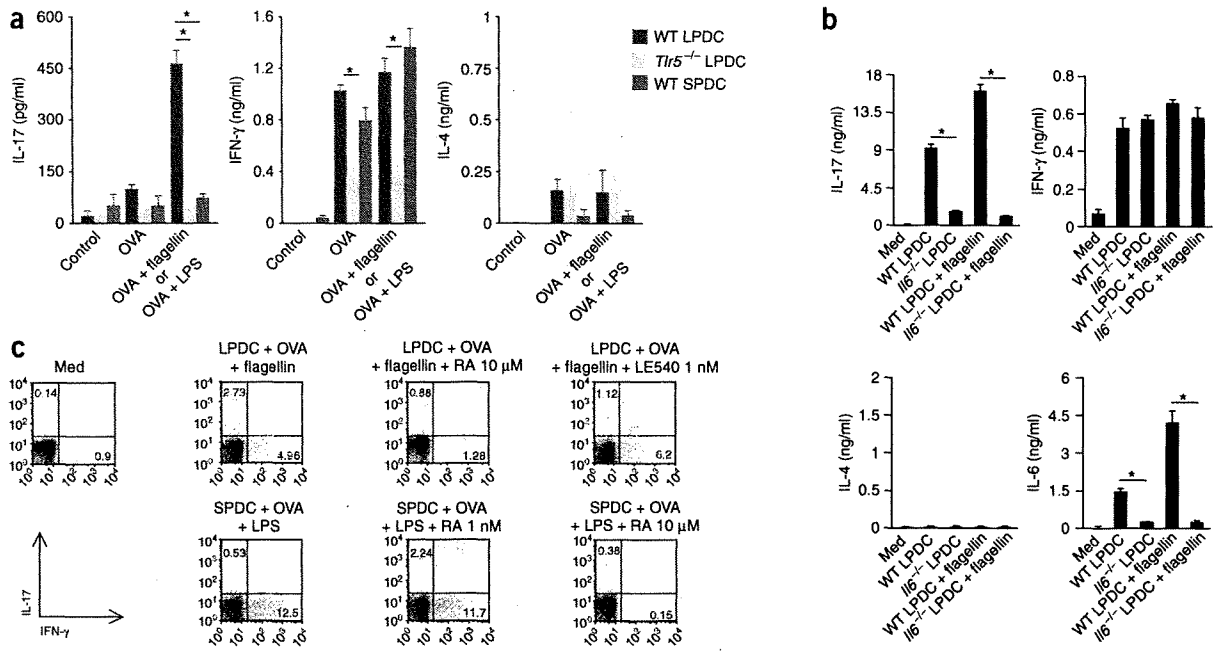

Figure 5 TLR5-dependent T_H-17 cell differentiation by CD11c^{hi}CD11b^{hi} LPDCs. (**a**) ELISA of IFN-γ, IL-17 and IL-4 in culture supernatants. CD11c^{hi}CD11b^{hi} LPDCs or SPDCs cultured for 12 h with OVA protein (100 μg/ml) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml) were 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

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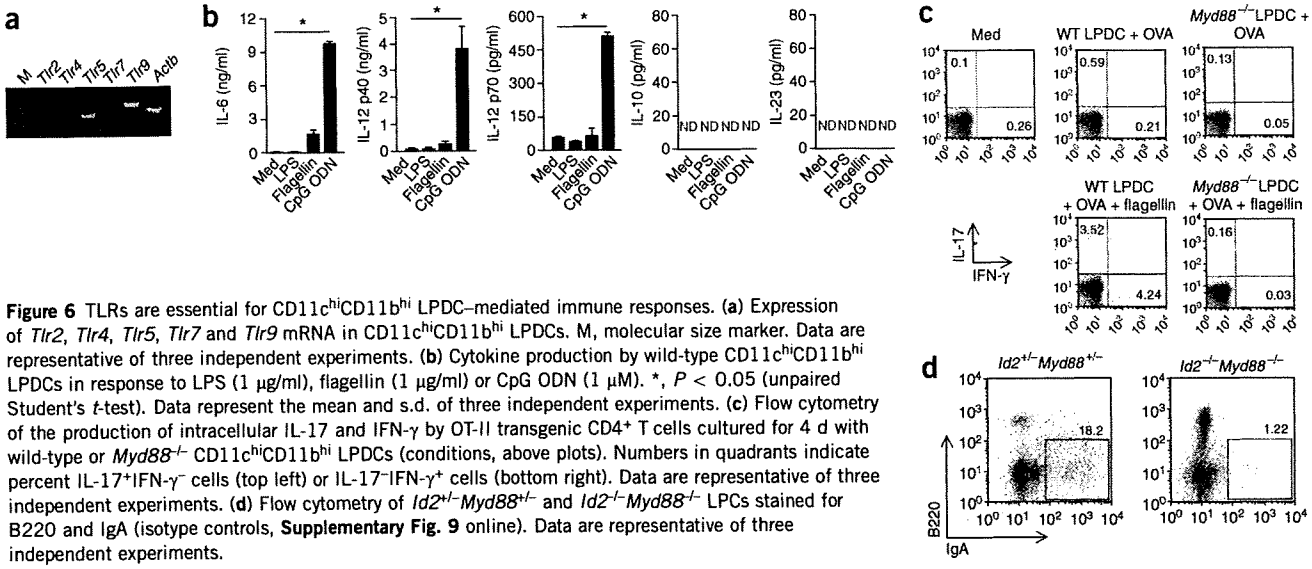

Figure 6 TLRs are essential for CD11c^{hi}CD11b^{hi} LPDC–mediated immune responses. (**a**) Expression of *Tlr2*, *Tlr4*, *Tlr5*, *Tlr7* and *Tlr9* mRNA in CD11c^{hi}CD11b^{hi} LPDCs. M, molecular size marker. Data are representative of three independent experiments. (**b**) Cytokine production by wild-type 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$_H$1 cell differentiation (**Fig. 5c** and **Supplementary Fig. 7** online). However, we suspected that this concentration of retinoic acid may have been too high, as plasma retinoic acid concentrations are usually on the order of 10 nM and retinoic acid efficiently enhances the expression of gut-homing receptors on CD8$^+$ 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$_H$1 cells, which suggested that retinoic acid from CD11c^{hi}CD11b^{hi} LPDCs is actually necessary for T$_H$-17 cell differentiation. In line with that observation, LPS-stimulated SPDCs induced T$_H$-17 cell differentiation to the same extent as flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs when cultured together with 1 nM retinoic acid, and 10 μM retinoic acid abolished T$_H$1 cell differentiation induced by LPS-stimulated SPDCs (**Fig. 5c** and **Supplementary Fig. 7**). Thus, retinoic acid at a low concentration acts as a positive regulator of T$_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$_H$1 cells, but it is not clear whether these adaptive immune responses are protective against bacterial infection. T$_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$_H$1 cells in the lamina propria (**Supplementary Fig. 8b**). Challenge of the immunized mice with oral OVA further increased the proportions of lamina propria T$_H$-17 and T$_H$1 cells. Similarly, immunization with *S. typhimurium* flagellin–loaded wild-type LPDCs resulted in a significant increase in the proportion of lamina propria T$_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

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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

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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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Effects of Probiotics on Allergic Rhinitis Induced by Japanese Cedar Pollen: Randomized Double-Blind, Placebo-Controlled Clinical Trial

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Key Words
Japanese cedar pollen · Allergic rhinitis · Probiotic · *Lactobacillus casei* · Fermented milk

Abstract
Background: *Lactobacillus casei* strain Shirota (LcS) has been found to exert antiallergic effects in animal experiments, but there is little information about its clinical effects in human patients with allergy. ***Methods:*** We performed a randomized double-blind, placebo-controlled study to investigate the effects of LcS in patients with allergic rhinitis triggered by Japanese cedar pollen (JCP). Participants were asked to drink fermented milk containing LcS (LcS group) or placebo (control group) for 8 weeks. Clinical symptoms and immunological parameters were compared between the two groups. ***Results:*** Symptom-medication scores (SMS) worsened in accordance with the increase in the amount of scattered JCP. In terms of the nasal and ocular SMS, there was no significant difference between the LcS group and the placebo group during the ingestion period. In the subgroup of patients with moderate-to-severe nasal symptom scores before starting the ingestion of test samples, supplementation with LcS tended to reduce nasal SMS. ***Conclusion:*** These results indicate that fermented milk containing LcS does not prevent allergic symptoms in patients sensitive to JCP, but may delay the occurrence of allergic symptoms in patients with moderate-to-severe nasal symptom scores.

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Introduction

Recently, patients with allergic rhinitis have been increasing in Japan, and the incidence of allergic rhinitis caused by Japanese cedar pollen (JCP) is estimated to be in the range of 10–15% among Japanese people [1]. This tendency could be a serious problem from the standpoint of socioeconomic aspects, because the allergic symptoms are unpleasant for patients and often cause various disturbances in their ordinary life during the particular season. Moreover, the medical bill for treating allergic symptoms is not negligible.

The general treatment of allergic rhinitis is administration of antihistamines, laser evaporation of the inferior turbinate, Vidian neurectomy and immunotherapy. These modalities are considered efficient but have some problems. Antihistamines sometimes cause side effects

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Table 1. Characteristics of the participants in the LcS group and the placebo group

Characteristics	LcS group	Placebo group	Significance
Number of patients (male:female)	55 (22:33)	54 (21:33)	NS
Age, years	39.3 ± 8.0	39.5 ± 10.9	NS
Total IgE, IU/ml	198.9 ± 273.8	160.0 ± 247.0	NS
Anti-JCP IgE, IU/ml	15.6 ± 21.5	14.0 ± 15.7	NS
Nasal SMS	1.33 ± 0.72	1.42 ± 0.88	NS
Ocular SMS	0.94 ± 1.01	0.95 ± 0.97	NS
Swelling of nasal mucosa	1.24 ± 1.00	1.20 ± 0.86	NS
Color of nasal mucosa	1.45 ± 0.90	1.52 ± 0.91	NS
Amount of mucus	0.89 ± 0.76	0.89 ± 0.66	NS
Nature of mucus	1.45 ± 1.32	1.50 ± 1.31	NS

NS = Not significant; SMS = symptom-medication score.

such as sleepiness, thirst or gastrointestinal disturbance, and their dose and timing of administration should be strictly controlled. Laser evaporation of the inferior turbinate, Vidian neurectomy and immunotherapy cause a great burden to the patient, such as hospitalization, or sometimes a long ambulatory treatment period. Under these circumstances, food products to prevent or improve allergic symptoms that are easily available in ordinary life are required. Thus, yogurt, tea and herbs have been shown to potentially relieve the allergic symptoms [2–5].

Supplementation with milk fermented with *Lactobacillus paracasei* 33, *L. acidophilus* L-92 or *Bifidobacterium longum* BB536 has been shown to suppress the subjective symptoms and may modulate immunological parameters in allergic rhinitis patients [6–9]. These findings support the opinion that stabilization of the intestinal microflora by administration of probiotics may prevent the development of allergic rhinitis.

L. casei strain Shirota (LcS) suppresses the IgE production of splenocytes by enhanced interleukin 12 secretion by macrophages in vitro [10], and its administration prevents the elevation of the IgE level and induction of anaphylactic symptoms after sensitization with ovalbumin in animal models [11, 12]. Therefore, it is worthwhile examining whether LcS could improve allergic symptoms in humans. We evaluated the effect of fermented milk containing LcS in patients with allergic rhinitis to JCP in a randomized double-blind, placebo-controlled study. This report shows that ingestion of fermented milk containing LcS did not prevent clinical symptoms or abnormal immunological parameters in patients allergic to JCP, but may delay the occurrence of subjective symptoms in patients with moderate-to-severe nasal symptom scores.

Materials and Methods

Subjects

To carry out this study, we recruited participants enrolled for human studies in the nontreated subject bank of Soiken Inc. (Osaka, Japan), which is an organization for evaluating the functions of foods or food-derived materials in humans. We explained the aim and protocol of this study, asked if they were willing to participate and screened for subjects having specific IgE for JCP by a scratch test using allergen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) and a radioallergosorbent test. Exclusion criteria were as follows: use of antihistamines or antiallergic medication at the time of the screening test; any recent history of acute rhinitis, sinusitis, nasal polyp, hypertrophic rhinitis, septal deformity or asthma; severe disorder of the liver, kidney, heart, respiratory organs, endocrine glands or metabolism; treatment with hyposensitization therapy; frequently drinking dairy products containing lactic acid bacteria, and cow's milk allergy. The study was performed in accordance with the Declaration of Helsinki and approved by the local ethics committee, which is independent of Soiken Inc. Written informed consent was obtained from all the participants.

Study Design

The study was performed in a randomized double-blind, placebo-controlled manner. Experiments were performed from January 22 to April 15, 2005. Randomization was performed by doctors, who were not involved in this study design. All of the enrolled subjects were randomly assigned to the LcS group or the placebo group according to computer-generated permuted-block randomization. There was no obvious difference in the two groups (table 1). The LcS group drank fermented milk containing LcS (4×10^{10} CFU/80 ml) and the placebo group drank unfermented milk. The composition of fermented milk containing LcS and placebo was the same except that the placebo did not contain LcS, and the lactic acid level of placebo was adjusted to the level of fermented milk containing LcS. Before delivery to the participants, we confirmed that the fermented milk contained more than 5×10^8 CFU/ml of LcS, and both fermented milk and placebo had no contamination with other bacteria.

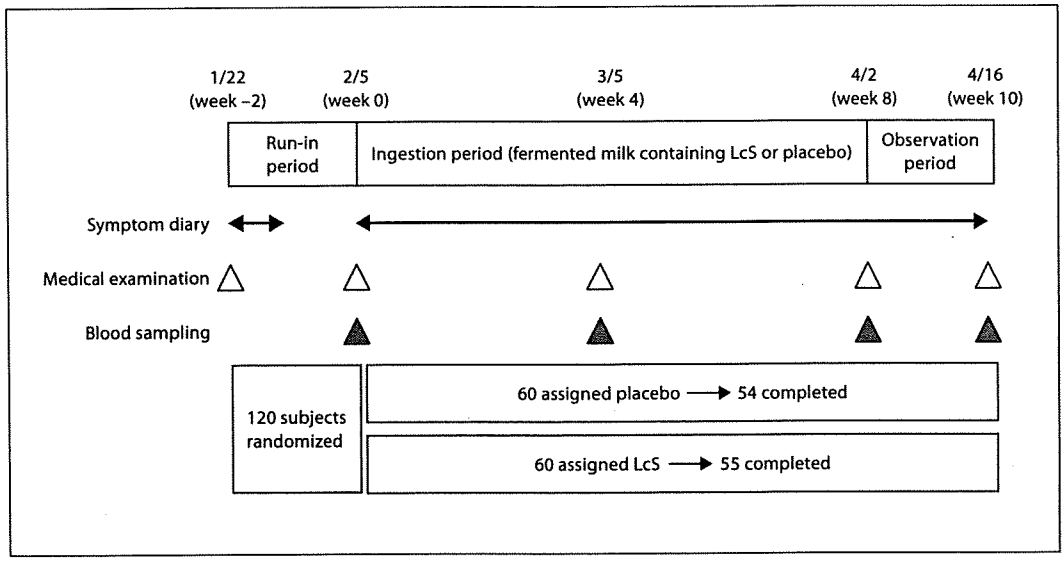

Fig. 1. Study protocol. The clinical trial was carried out from January 22 to April 15, 2005. Participants were asked to drink fermented milk containing LcS or placebo for 8 weeks, and to record their symptoms in a diary every day. Medical examination was conducted 5 times, and blood samples were taken 4 times during the study.

To assure the viability of LcS, we prepared fermented milk every week during the study period. Furthermore, we checked the acidity and sugar level in LcS-containing fermented milk and confirmed that these values were within standardized levels. We did not check the intestinal microflora in the volunteers in this study. However, it has already been verified that LcS can be detected in feces after supplementation of LcS-containing fermented milk [13–15]. We asked all the participants not to change their ordinary lifestyle during the study. The participants drank 80 ml of placebo or fermented milk containing LcS daily for 8 weeks. The schedule of the study is shown in figure 1. Participants were asked to record their nasal and ocular symptoms and medication in a diary during the study period. Moreover, they underwent medical examination by an otolaryngologist 5 times during the study.

Evaluation of Symptoms and Medical Examination of Subjects

The scores of nasal and ocular symptoms have been defined by the Japanese Society of Allergology [1, 16]. Briefly, sneezing, runny nose, stuffy nose, itchy eyes and watery eyes were each scored from 0 to 4 according to the severity of symptoms by the participants, and the medication score was estimated based on the efficacy of medicines (table 2). The medication score, which was described in the guidelines of the Japanese Society of Allergology, is determined by medication usage (table 2). The clinical condition of the nasal cavity (swelling and color of nasal mucosa, amount and nature of mucus) was scored from 0 to 3 for each feature according to the severity by the otolaryngologist (table 3). The symptom-medication score (SMS) was calculated by summing the symptom score and the medication score.

Table 2. Symptom score and medication score for evaluating the subjective symptoms

a Symptom score

Score	Sneezing[a]	Runny nose[b]	Stuffy nose	Itchy eyes	Watery eyes
0	0	0	none	none	none
1	1–5	1–5	mild	mild	mild
2	6–10	6–10	moderate	moderate	moderate
3	11–20	11–20	severe	severe	severe
4	>21	>21	violent	violent	violent

b Medication score

Score	Medicine
1	oral antihistamine, oral histamine release inhibitor, nose or eye drops (without steroids)
2	local administration of steroids
3	oral antihistamine plus local administration of steroids

Sneezing = Average number of sneezing attacks in a day; runny nose = average number of times patient blew nose in a day.

Table 3. Standard for doctors of otolaryngology to assess the nasal cavity

Score	Swelling of mucosa	Color of mucosa	Amount of mucus	Nature of mucus
0	none	normal	none	none
1	middle concha observable	light red	adhesion level	purulent
2	between 1 and 3	red	between 1 and 3	viscous
3	middle concha unobservable	bluish	filled	aqueous

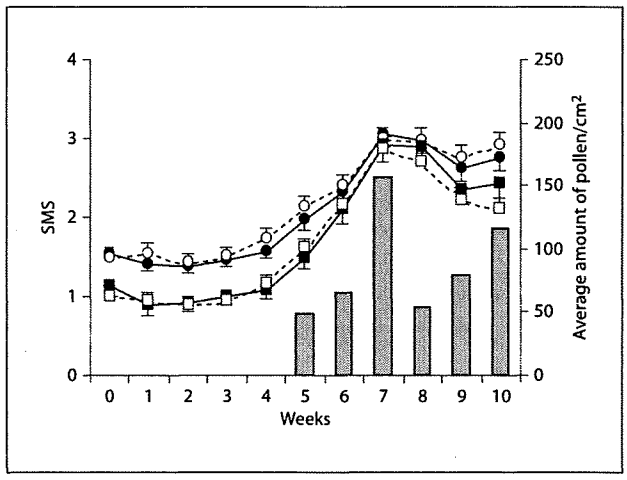

Fig. 2. Nasal and ocular SMS throughout the study period. Nasal SMS in the LcS group (●) and placebo group (○) and ocular SMS in the LcS group (■) and placebo group (□) are shown as means ± SE. Filled columns show the average amount of pollen (JCP and hinoki pollen) scattered in the area where the studies were carried out.

Blood Examination

Blood samples were collected 4 times during the study, and anti-JCP IgE, eosinophil number, eosinophil cationic protein (ECP) and the balance of Th1 cells to Th2 cells (Th1/Th2 ratio) were determined. The anti-JCP IgE level was evaluated by the radioallergosorbent test. The ECP level was measured by radioimmunoassay (Unicap system; Roche Diagnostics KK, Sweden). Peripheral blood mononuclear cells were stimulated with phorbol myristate acetate plus ionomycin for 4 h in the presence of brefeldin A, and stained with anti-CD4 antibody. After cells had been fixed and permeabilized, the accumulated γ-interferon and interleukin 4 in CD4+ T cells were stained and measured by a flow cytometer. The ratio of γ-interferon+ CD4+ T cells to interleukin-4+ CD4+ T cells was expressed as Th1/Th2 balance.

Statistical Analysis

SMS were averaged each week. Differences in SMS and description of the nasal mucosa between the groups were evaluated by the Mann-Whitney U test. Differences in immunological parameters were assessed by the unpaired Student's t test. Data were analyzed using SPSS software (version 11.5, SPSS Inc., Chicago, Ill., USA).

Results

Scattering of JCP

The Osaka Prefectural Institute of Public Health and the Japan Weather Association reported that much JCP was scattered in the spring of 2005, and the level of JCP reached 10–30 pollen/cm^2 on the first 10 days of March (4–5 weeks after the start of ingestion), being maximal (approx. 600 pollen/cm^2) in the last 10 days of March (7 weeks). Thereafter, the amount of scattered JCP gradually declined, although it was still detected at more than 10–30 pollen/cm^2 until the beginning of April (9 weeks). Furthermore, hinoki pollen started to scatter from the beginning of April, causing a biphasic change in the average amount of total pollen in the spring of 2005 (fig. 2).

Study Population

One hundred and twenty subjects were enrolled, but 11 subjects declined to take part in the study for personal reasons. As a result, 109 subjects (54 subjects in the placebo group and 55 in the LcS group) completed the study and their data were analyzed (fig. 1). There was no difference between the groups in terms of age, total and anti-JCP IgE level, and severity of allergic symptoms before the study (table 1).

Subjective Symptoms

Nasal and ocular SMS began to rise during the first 10 days of March in 2005 (4 weeks after the start of ingestion) and increased in association with the increase in scattered JCP. Deterioration of nasal SMS was delayed 1 week in the LcS group compared with the placebo group, but the difference in nasal SMS between the groups was not significant during the study (fig. 2). However, when the patients were divided into two categories ('mild' and

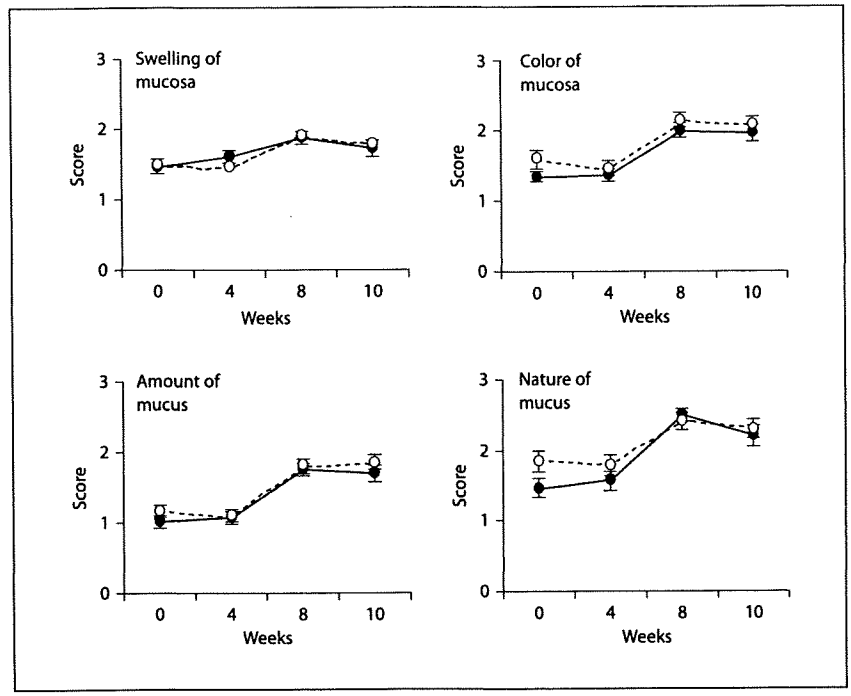

Fig. 3. Score of condition of nasal mucosa throughout the study. Data are presented as means ± SE. ● = LcS group; ○ = placebo group.

'moderate-to-severe') based on the nasal symptom score before the study, the nasal SMS in moderate-to-severe cases in the LcS group was lower at 4 and 5 weeks than in the placebo group (LcS group n = 13; placebo group n = 11; data not shown).

Medical Examination of Nasal Cavity

Scores of swelling and redness of the nasal mucosa, and the amount and nature of mucus deteriorated in association with the increase in scattered JCP. However, none of these scores differed between the groups (fig. 3).

Blood Examination

Immunological parameters associated with allergic symptoms increased in response to the amount of scattered JCP. Compared with the value before ingestion, the ECP level rose by 4 weeks and the anti-JCP IgE level and eosinophil number increased by 8 weeks. No difference was detected in immunological parameters between the two groups during the study (fig. 4). We divided the participants into mild cases and moderate-to-severe cases based on the nasal symptom score before the study and performed the statistical analysis, but there was no significant difference between the two groups (data not shown).

Side Effects

Ten subjects suffered from a cold, 3 subjects developed diarrhea, and 1 subject vomited during the study. All of these disorders were transient and there was no concern that they were related to the ingestion of fermented milk containing LcS (data not shown).

Discussion

We investigated the effect of fermented milk containing LcS on allergic symptoms triggered by JCP in a randomized double-blind, placebo-controlled study. The results showed that nasal and ocular SMS worsened in accordance with the increase in scattered JCP, confirming that the subjective symptoms of allergic rhinitis appear swiftly in response to scattered JCP. We have also performed a similar study in the spring of 2004 when a very small amount of JCP was scattered. Most patients did not experience any problem related to allergic symptoms, and nasal and ocular SMS in the participants did not rise in the spring of 2004 (data not shown). Taken together, these observations demonstrate that subjective symptoms are good parameters to assess allergic rhinitis during the JCP season.

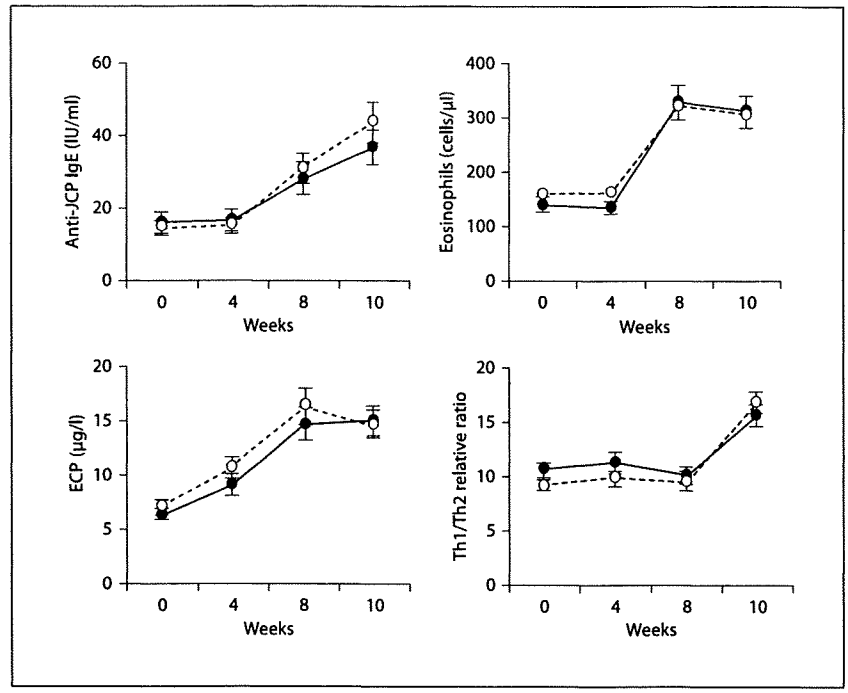

Fig. 4. Immunological parameters throughout the study period. Data are presented as means ± SE. ● = LcS group; ○ = placebo group.

Nasal and ocular SMS were not different between the LcS group and the placebo group. These results show that supplementation with fermented milk containing LcS during the JCP season does not prevent the exacerbation of seasonal allergic rhinitis. When the subjects were divided into two subgroups based on the severity of nasal symptom score before the start of ingestion, nasal SMS of the participants with moderate-to-severe nasal symptom scores was lower in the LcS group than in the placebo group at 4 weeks and 5 weeks, indicating that LcS may delay the onset of allergic symptoms in patients with a moderate-to-severe condition. In previous studies, we found that a daily drink of 80 ml of fermented milk containing LcS caused recovery of natural killer cell activity in humans [17–19]. Therefore, we considered that 80 ml of fermented milk containing LcS could be sufficient for modulating human immune function. However, it remains to be elucidated whether more than 80 ml may improve the results in placebo-controlled trials using participants with moderate-to-severe nasal symptoms in future investigations.

The clinical effects of probiotics on allergic rhinitis have been examined in several studies. Fermented milk containing *L. paracasei* LP-33 reduced the subjective symptoms in patients with perennial allergic rhinitis more efficiently than fermented milk containing *Streptococcus thermophilus* and *L. bulgaricus* [6]. Fermented milk containing *L. acidophilus* L-92 improved the subjective symptoms in not only perennial allergic rhinitis, but also Japanese cedar pollinosis, compared with heat-treated milk, although the immunological parameters were not different between the L-92 group and the placebo group [7, 8]. Furthermore, the clinical symptoms in Japanese cedar pollinosis were relieved in patients ingesting yogurt fermented with *B. longum* BB536, *S. thermophilus*, and *L. delbrueckii* compared with those ingesting yogurt fermented with *S. thermophilus* and *L. delbrueckii*, and the immunological parameters were modulated by *B. longum*-BB536-fermented yogurt [9]. These results suggest that some probiotic strains and/or their fermentation products are responsible for improvement of allergic rhinitis. In contrast, supplementation with capsules containing *L. rhamnosus* GG did not improve the clinical symptoms of birch-pollen-allergic patients [20]. Therefore, further investigation is required to elucidate the antiallergic effects of probiotics on allergic rhinitis. Viljanen et al. [21] have reported that administration of *L. rhamnosus* GG did not improve the clinical scores in infants with food allergy, but this probiotic was considered effective for the improvement of symptoms in the IgE-sensi-

tized subgroup. Our results also suggest that supplementation with LcS might delay the onset of allergic symptoms in subjects with moderate-to-severe symptom scores. Altogether, the effect of probiotics on allergic rhinitis may vary dependent on the disease condition.

The effects of probiotics to modulate immunological parameters associated with allergic symptoms should be elucidated. It was reported that LcS suppressed IgE production in vitro and reduced the antiovalbumin IgE level in wild-type mice and transgenic mice expressing ovalbumin-specific T-cell receptor [10–12]. However, no difference was detected in immunological parameters between the LcS group and the placebo group in this clinical trial. Probiotics may possibly improve subjective symptoms even if immunological parameters such as the allergen-specific IgE level or Th1/Th2 imbalance are not normalized. In that sense, it is of great interest that supplementation with *L. rhamnosus* 19070–2 and *L. reuteri* DSM stabilized the intestinal barrier function and decreased gastrointestinal symptoms in children with atopic dermatitis [22]. Moreover, involvement of natural killer T cells and regulatory T cells in the induction and control of allergic responses has been proposed [23, 24]. Therefore, other mechanisms besides suppression of IgE production or normalization of Th1/Th2 imbalance could be involved in the antiallergic activity exerted by probiotics in humans.

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