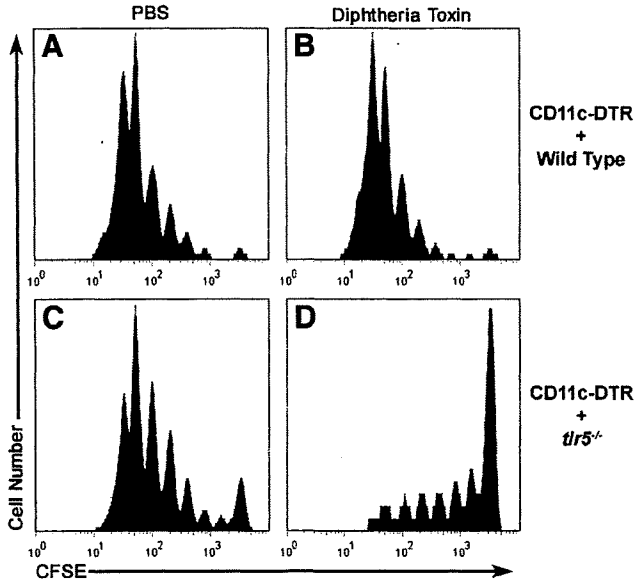

FIGURE 6. Deletion of $tlr5^{+/+}$ CD11c$^+$ cells severely impairs OVA-specific CD4$^+$ T cell proliferation in mice immunized with flagellin-OVA fusion protein. Irradiated C57BL/6 mice were reconstituted with 50% CD11c-DTR bone marrow and 50% WT (*A* and *B*) or 50% $tlr5^{-/-}$ BM (*C* and *D*) and rested for 12 wk. Chimera mice then received 3×10^6 CD4$^+$-enriched, CFSE-labeled CD4$^+$CD90.1$^+$ OT-II cells and were injected with PBS (*A* and *C*) or DT (*B* and *D*) 1 day following cell transfer. Eighteen hours after toxin treatment mice were immunized i.m. with 1×10^{-11} mol of flagellin-OVA and sacrificed 3 days following immunization. Proliferation by the OT-II population in the draining, popliteal lymph node was compared based on CFSE dilution. Plots are gated on CD4$^+$CD90.1$^+$ cells. The data in this experiment were obtained from four mice per group.

of flagellin-OVA or PBS. Mice were sacrificed 3 days later, and CFSE dilution was compared among OVA-specific cells recovered from the draining lymph nodes of mice from each group. In confirmation of the results presented in Fig. 6, flagellin-OVA induced T cell proliferation was dramatically reduced in immunized $tlr5^{-/-}$ mice relative to wild-type mice (Fig. 7).

To address the individual roles of enhanced Ag uptake via TLR5 and TLR5 signaling in the effect of flagellin-OVA, we evaluated the proliferation of OT-II cells in immunized $MyD88^{-/-}$ mice. Since $MyD88^{-/-}$ mice have normal TLR5 expression, if Ag targeting is sufficient to fully account for the enhanced potency of flagellin-OVA vs flagellin plus OVA, then OVA-specific cells recovered from $MyD88^{-/-}$ mice should exhibit Ag-specific proliferation that is equivalent to that observed in wild-type mice. However, if TLR5 signaling is critical, then OVA-specific cells recovered from $MyD88^{-/-}$ mice should exhibit lower levels of Ag-specific T cell proliferation than observed in wild-type mice. Like the situation in $tlr5^{-/-}$ mice, OT-II proliferation was quite low in MyD88$^{-/-}$ mice (Fig. 7*C*). Although these results are consistent with the hypothesis that the enhanced effect of immunization with flagellin-OVA fusion protein is dependent on signaling through TLR5, it is possible that the lack of MyD88 might have a negative effect on Ag processing that is independent of TLR5 (37, 38). To address this question, we used the assay for in vivo T cell Ag-specific clustering described in Fig. 2 to assess the ability of OT-II cells to interact with DC in $MyD88^{-/-}$ mice. We found that 9% of adoptively transferred OVA-specific T cells were engaged in clusters in the draining lymph nodes of $MyD88^{-/-}$ mice 24 h after immunization (as opposed to 24% in wild-type mice; Fig. 2). By comparison, <2% OVA-specific T cells were engaged in

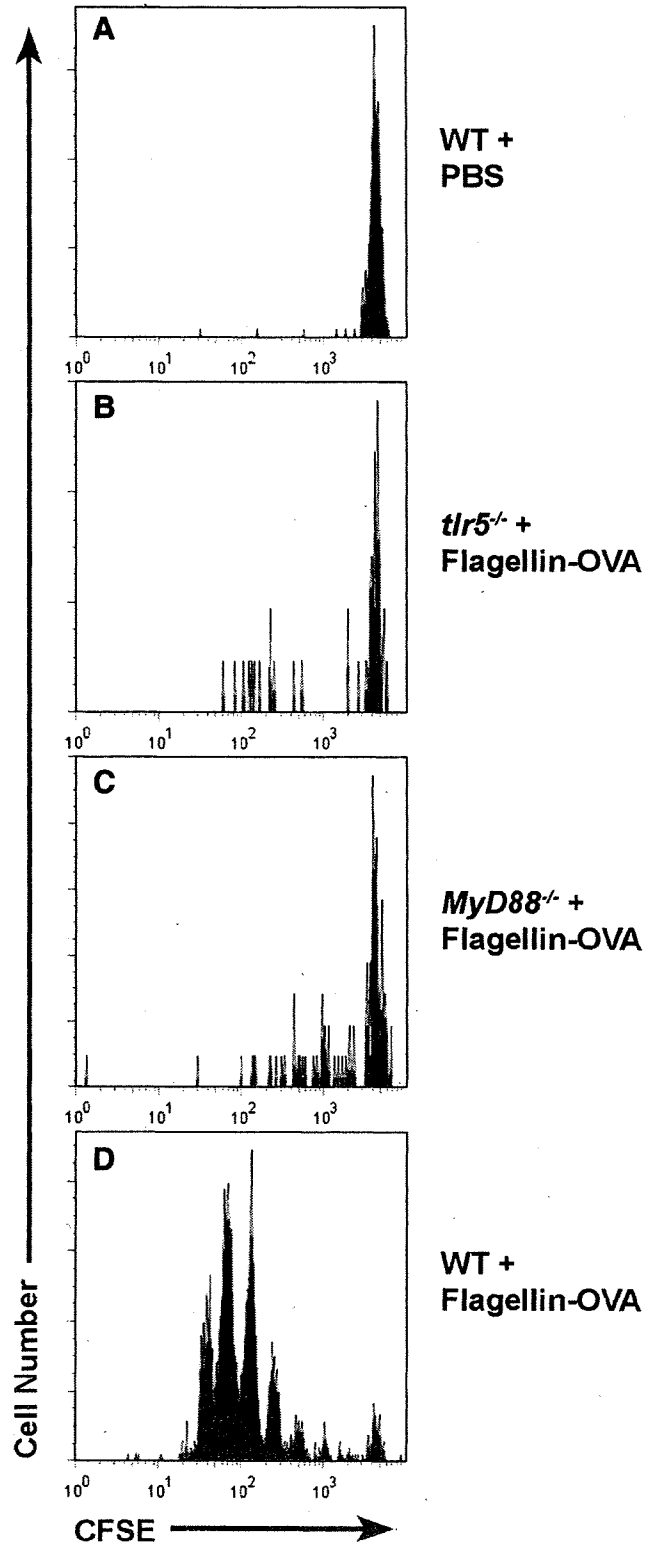

FIGURE 7. TLR5 and MyD88 are necessary for the in vivo adjuvant effect of flagellin. C57BL/6 (*A* and *D*), $tlr5^{-/-}$ (*B*), and $MyD88^{-/-}$ (*C*) mice were i.v. injected with 1.5×10^6 CD4$^+$-enriched CFSE-labeled, CD4$^+$CD90.1$^+$ OT-II T cells. Twenty-four hours later, mice were immunized i.m. with 1×10^{-11} mol of flagellin-OVA fusion protein. Mice were sacrificed 3 days following immunization, and proliferation by the OT-II population in the draining, popliteal lymph node was compared based on CFSE dilution. Plots are gated on CD4$^+$CD90.1$^+$ cells. The data in this experiment were obtained from four mice per group.

clusters in *MyD88*$^{-/-}$ immunized with just OVA. These findings are consistent with the hypothesis that the enhanced effectiveness of flagellin-OVA as opposed to flagellin plus OVA is due to enhanced Ag uptake via TLR5 as well as signaling through MyD88. However, it is important to emphasize that the precise contributions of each of these mechanisms must await future studies in which we are able to measure the activation of the DC within the lymph node itself.

Discussion

The results presented in this study are consistent with the conclusion that the adjuvant effect of flagellin is dependent, at least in part, on a high-affinity interaction with TLR5 on CD11c$^+$ cells that facilitate extremely efficient uptake of Ag (when the Ag is part of a flagellin fusion protein) via TLR5 (Figs. 6 and 7). On the basis of the retention of normal patterns of T and B lymphocyte staining following DT treatment (Fig. 5), we believe that the DT-sensitive CD11c$^+$ cell that is required for the adjuvant effect of flagellin is a DC. The observation that immunization with flagellin-OVA is more effective at promoting an Ag-specific immune response than immunization with equimolar doses of flagellin and OVA given as separate proteins is clearly consistent with this conclusion and can be explained by two actions of flagellin-enhanced efficiency of Ag uptake via TLR5 and signaling via TLR5 that promotes DC activation. Our results (Figs. 5 and 6) are consistent with the hypothesis that both of these actions are important in the overall adjuvant effect of flagellin in the context of a flagellin-Ag fusion protein. On the basis of the observation that 24 h following immunization with flagellin-OVA, 9% of OT-II cells in *MyD88*$^{-/-}$ are in clusters compared with 24% in wild-type mice, we estimate that approximately one-third of the adjuvant effect of flagellin results from Ag targeting to DC and two-thirds from signaling through TLR5 and MyD88. However, as noted previously, additional studies are required to quantitate the contributions of each mechanism to the overall stimulatory effect of DC on T cell activation. MyD88 deficiency has been shown to negatively affect phagocytosis (38) and phagosome maturation (37). However, on the basis of the comparisons with our observations, processing of Ag acquired by TLR5-mediated endocytosis appears to be less dependent on MyD88 function than does processing of Ag acquired by phagocytosis.

It is important to emphasize that flagellin can also function as an adjuvant when the Ag is not part of the flagellin protein (9, 11, 39), but the response requires significantly higher doses of Ag and flagellin (~10-fold; Fig. 4). Given the extremely high affinity of flagellin for TLR5 (25, 40), the uptake of flagellin-OVA via TLR5-dependent endocytosis is likely to be far more efficient than the uptake of OVA by itself. As noted earlier, others have shown that targeting Ag for uptake by DC can enhance the Ag-specific in vivo immune response (33, 34). If as expected, uptake and processing of flagellin-OVA fusion protein is much more efficient by TLR5-expressing DC than uptake of OVA alone, the enhanced response to fusion protein could simply result from concentration of Ag by TLR5$^+$ DC and presentation of a greater number of MHC class II molecules loaded with the cognate peptide as opposed to enhanced uptake and TLR5 signaling. Immunization with high doses of OVA alone can trigger proliferation of OVA-specific CD4$^+$ T cell. Thus, if the adjuvant effect of flagellin fused to Ag is mediated predominantly through enhanced uptake and processing following TLR5 ligation and not signaling via TLR5, *tlr5*$^{+/+}$*MyD88*$^{-/-}$ mice should generate a significantly more robust response to flagellin-OVA than *tlr5*$^{-/-}$*MyD88*$^{+/+}$ mice. Our finding that *MyD88*$^{-/-}$ and *tlr5*$^{-/-}$ mice respond similarly to immunization with flagellin-OVA (Fig. 7) provides strong evidence in support of the conclusion that TLR5 and MyD88 are necessary for the adjuvant effect of flagellin and that Ag concentration by TLR5$^+$ APC may not fully account for the adjuvant effect of flagellin. Our experimental approach has relied heavily on the OVA-specific TCR transgenic OT-II cells, a widely accepted model for studying CD4$^+$ T cell biology. Several groups have demonstrated that the increased precursor frequency in adoptive transfer model systems impacts the dynamics of the immune response (41–43); thus, it is possible that polyclonal wild-type cells could exhibit slightly different response than seen in this model system.

In confirmation of other reports (15, 44), we have shown that flagellin does not significantly activate murine BMDC. Although others have arrived at the opposite conclusion (14), those results were generated using doses of flagellin 30–100× in excess of what we have found to be a maximally active concentration (10^{-10} M) and could result from contaminating nucleic acids not removed by endotoxin depletion. Since the responsiveness to flagellin of monocytes (45) and human myeloid-derived DC (C. L. Hickman, J. T. Bates, and S. B. Mizel; unpublished observations) are dependent on their differentiation state, it is quite likely that the inability of murine BMDC to respond to flagellin reflects their degree of maturation rather than a general property of mature murine DC. Uematsu et al. (24, 46) identified a population of TLR5$^+$ DC in the lamina propria of the murine small intestine that is clearly responsive to flagellin. However, Salazar-Gonzalez et al. (17) concluded that flagellin does not directly activate murine splenic DC. Our results as well as those of these investigators support the idea that responsiveness to flagellin varies among DC populations and that the environment in which DC terminally differentiate can significantly modify or induce their responsiveness to flagellin.

Sanders et al. (47) recently reported that flagellin is capable of promoting Ag-specific humoral immunity by a mechanism that is independent of TLR5. The flagellin doses used in their studies are markedly higher than those used in our studies (0.8 μg in our studies vs 50 μg used by Sanders et al.). Indeed, the doses used by Sanders et al. (47) are higher than what is required to drive a maximal response in nonhuman primates (13) and likely reflects a nonspecific effect of high-dose flagellin. It should be noted, however, that Sanders et al. also found that cytokine production in the innate immune response and activation of DC was severely limited in TLR5-deficient mice.

Our findings, in conjunction with what was previously known about flagellin, are consistent with the conclusion that the potent adjuvant effect of flagellin results from the synergy of three distinct processes: direct activation of TLR5$^+$ DC (Figs. 6 and 7), cytokine and chemokine production by non-DC (12, 48–50), and activation of the vascular endothelium (51). Binding of flagellin by TLR5$^+$ DC leads to activation of NF-κB-regulated genes. A number of these genes are critical to mounting an effective immune response. Several reported outcomes of NF-κB activation in DC include enhanced Ag processing and presentation (52, 53), up-regulation of comstimulatory molecules (53), and cytokine (53–55) and chemokine (56) production. Notably, NF-κB activation in human DC results in up-regulation of ICAM-1, ICAM-3, and LFA-1, which are important molecules in facilitating DC-T cell interactions (53). Collectively, activation of NF-κB can have a significant adjuvant effect on the activity of DC in vivo (57). Consequently, in our system, DC that have been activated by flagellin and simultaneously pulsed with cognate Ag are especially effective at promoting an Ag-specific immune response. However, crucial to this outcome is the activation of cytokine and chemokine production by non-DC and also of the vascular endothelium. Activation of these cell populations is likely responsible for the increased flux of lymphocytes into draining lymph nodes soon after immunization

(Fig. 3 and Ref. 11). An increase in the number of lymphocytes entering the lymph node and possibly prolonged retention in the node maximize the likelihood that Ag-specific lymphocytes will encounter their cognate Ag. For T cells, presentation of that Ag by an activated DC ensures that they will receive Ag and costimulation sufficient to mount an immune response.

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Disclosures

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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

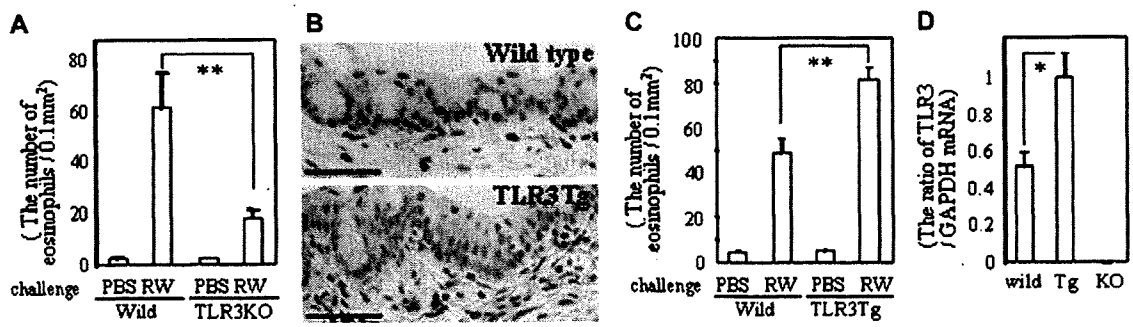

FIG 1. **A**, Eosinophil infiltration in *TLR3*KO mice. **B**, Eosinophil infiltration into the conjunctiva of ragweed-challenged wild-type and *TLR3*Tg mice was detected with Luna's method. Scale bars = 50 μm. **C**, Eosinophil infiltration in *TLR3*Tg mice. **D**, *TLR3* mRNA expression in eyelids. 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. $**P < .01$.

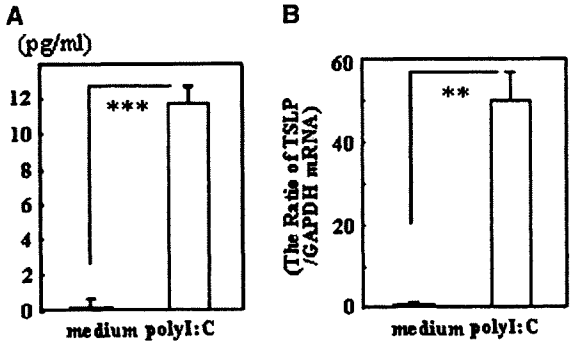

FIG 2. **A**, TSLP production. **B**, *TSLP* mRNA expression. The *y-axis* shows the increase in specific mRNA over that seen in medium samples. Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 μg/mL polyI:C and then incubated for 24 (Fig 2, *A*) or 6 (Fig 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

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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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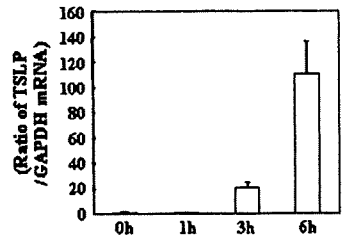

FIG E1. Increased *TSLP* mRNA expression by TLR3 stimulated with polyI:C. The *y-axis* shows the increase in specific mRNA over 0-hour samples or medium samples. The x-axis shows the time after stimulation. The data are presented as the means ± SEMs of 3 samples.

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ROLE OF TOLL-LIKE RECEPTORS IN PERSISTENT INFLAMMATION IN NASAL AND PARANASAL SINUS MUCOSA

INTRODUCTION

Bacterial infection and its degradation product such as LPS and teichoic acid, has been postulated to induce nasopharyngeal or tubotympanal inflammation and type-I allergic reaction could coincide as a prolongation factor. Most recently, the immune reaction can be categorized as an innate and acquired immunity, and Toll-like receptors expressed in various cells of mucosal linings, plays an important role in a defense mechanism against bacterial infection. However, on the other hand, once ostium or tubal blocking is achieved by mucosal swelling, paranasal sinus or middle ear cleft inflammation might become persistent. Therefore, from this standpoint, we attempted to investigate the distribution of Toll-like receptors in upper respiratory epithelial cells such as human cell lines or mast cells. From the functional aspects, in vitro study, the exact role of TLR2 and TLR4 in IL-8 and IL-15 production from epithelial cells was examined when these cells were stimulated with lipoprotein or LPS. Also, we examined in vitro Th2 type cytokine production of murine mast cells stimulated with cross-linking and modulation effects of LPS for cytokine synthesis in a murine allergic rhinitis model. In these protocols, antihistamines are examined to down regulate this cytokine production.

MATERIALS AND METHODS

1. Cells; Human respiratory epithelial cells; CCL30,CCL185 (ATCC). Human monocyte; U937(ATCC) Medium: DMEM with 10%FCS, RPMI1640 with 10% FCS.
   BMMCs were derived from femoral bone marrow cells of 6-week-old-Balb/c mice. After 3 weeks of culture with 10% Walter and Eliza Hall Institute (WEHI)-3- conditioned medium with as a source for IL-3, the cells were harvested for the experiments and consisted of more than 98% mast cells assessed by toluidine blue staining.
2. Cetirizine and tranilast: Cetirizine was manufactured by UCB (Brussels, Belgium) as the test drug. Tranilast was manufactured by Kissei Pharmaceutical Co., Ltd (Matsumoto, Japan).
3. Reagents: a-human TLR2, TLR4 and mouse IgG2a(eBioscience). Synthetic Lipid A was provided by Ono Pharmaceutics. Lipoprotein was provided by Bachem.
4. RNAanalysis: Expression of TLR2,3,4,6 and 9, expression of IL-15 and MyD88 was analyzed by Northern blot analysis. Northern blot analysis: Total cellular RNA was prepared using TRIzol reagent. Total cellular RNA was extracted from each cell culture. For RNA blotting, 5-15mg aliquots of total RNA were electrophoresed in agarose gels. RNAs were transferred to a nylon membrane. After ultraviolet-crosslinking, membranes were soaked in prehybridization solution and then incubated with (32P) aCTP-labeled probe in hybridization solution. The membranes were washed and then exposed to films. Expression of IL-15 was also analysed by ABI 7700. IL-15 mRNA load=(value of IL-15/value of GAPDH)X10^4
5. Luciferase assay: CCL185 cells were transiently transfected with 2mg of pGL3-NF-kB/Luc and 0.2 mg of pRL/SV40 by Lipofectamine according to the manufacture's instruction. Twenty-four hours after the transfection, some cells were pretreated with indicated chemicals for 30 min followed by the addition of Lipoprotein. After 8 hours incubation with Lipoprotein, cells were lysed, and the luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Toyo Ink).
6. DNA-binding assay: After 0.5 hour incubation with lipoprotein 1mg/ml, cells were lysed. NF-kB activity was measured by using NF-kB p50 Transcription Factor Assay Kits(ACTIVE MOTIF).
7. Western blot assay: Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and phosphorylation was detected by autoradiography.
8. Northern blot analysis:Total cellular RNA was extracted from each cell culture. For RNA blotting, 5-15mg aliquots of total RNA were electrophoresed in agarose gels. RNAs were transferred to a nylon membrane. After ultraviolet-crosslinking, membranes were soaked in prehybridization solution and then incubated with (32P) aCTP-labeled probe in hybridization solu-

tion. The membranes were washed and then exposed to films.

9. ELISA assay: Concentration of IL–15 in the culture supernatants of respiratory epithelial cells were measured by commercial ELISA kit (GT) according to the manufacture's instruction.
10. Flow cytometric analysis: The cells were stained with FITC-and PE conjugated mAb. FITC-aTLR4, PE-aTLR2mAb and mice IgG2awere used. The stained cells were analyzed by a FACSCalibur(Becton Dickinson).
12. Determination of cytokine production: Cytokines in culture supernatants were measured individually by an ELISA (R&D Systems).
13. Statistical analysis: The statistical significance of data was determined by Student's *t*-test. A value of $p<0.05$ was taken as significant.

RESULTS

Distribution of TLRs in human epithelial cells in nasopharyngeal mucosae and involvement of IL-15 in allergic reaction

The northern blot assay and RT-PCR data are shown in regard to TLR distribution for cultured human nasal epithelial cells , and somehow, we could not detect any TLR4 and TLR 9 expression at messenger RNA level. As result, respiratory epithelial cells constitutively expressed messenger RNA for TLR2, 3, 6, but not for TLR4 and TLR9. In northern blot analysis, IL-15 mRNA was strongly expressed after lipoprotein stimulation. But in contrast, it was not found after lipid stimulation as a ligand of TLR4. IL-15 concentration in the supernatants of CCL185 was also upregulated after lipoprotein stimulation in a dose-dependent manner.

Lipoprotein induced IL-15 and IL-8 production of respiratory epithelial cells, which strictly depend on TLR2 (Fig.1). Lipoprotein induced IL-15 production of respiratory epithelial cells was abolished by NF-kB inhibition (Fig.2). Lipoprotein-mediated IL-8 production in respiratory epithelial cells was abolished with NF-kB inhibition by Oxatomide (Fig.3).

Inhibitory effect of antihistamine on cytokine production from mast cells in vitro with cross-linking with IgE and Antigens

In vitro culture of Bone marrow-derived mast cells(BMMCs) indicated that allergen-induced IL-5 production from mast cells was downregulated by Cetirizine pretreatment (Fig.4). But, it was not influenced by Tranilast pretreatment. Cetilizine did not suppress IL-5 production from mast cells , if anti-DNP IgE on BMMCs was crosslinked with a high dose of DNP antigens.

Effect of Lipopolysaccharide(LPS) on murine allergic rhinitis model at the eliciting phase

Mast cells which is the key player at the eliciting phase of allergic rhinitis, have been reported to

Figure 1

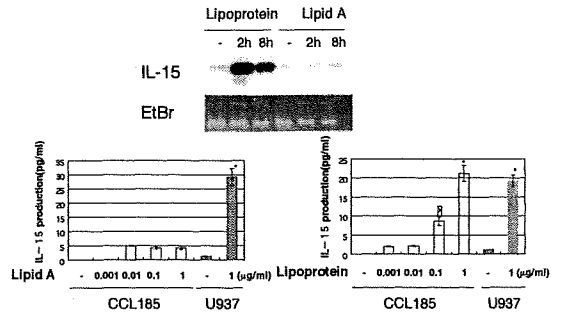


Figure 2

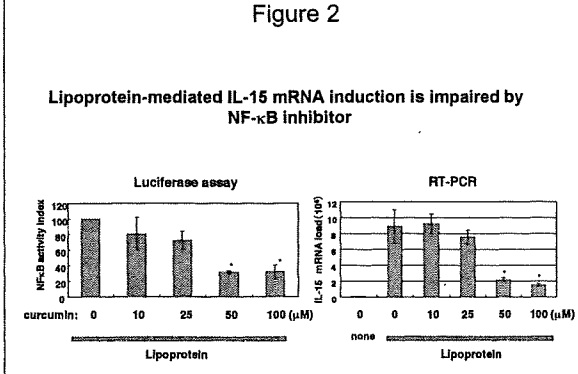


Figure 3

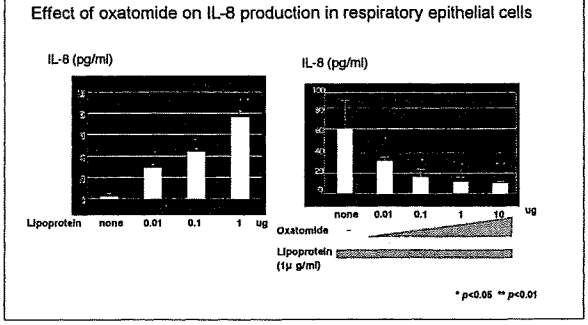


Figure 4

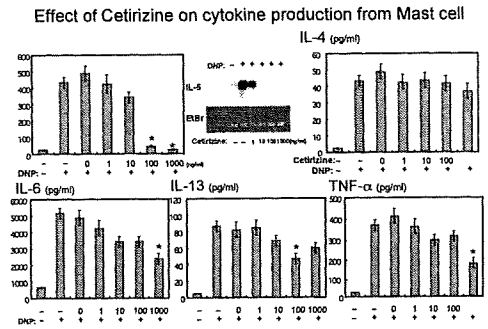
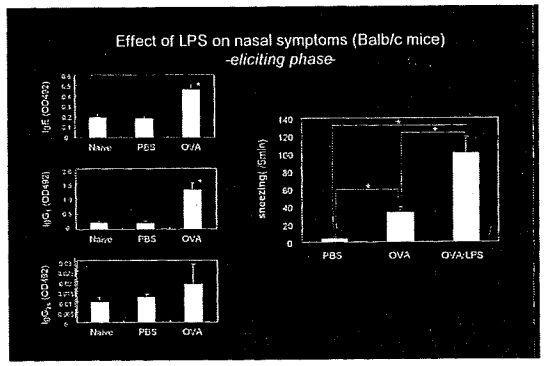


Figure 5



produce Th2 cytokines *in vitro* with LPS stimulation via TLR4 , but *in vivo* study remains to be performed. Therefore, we investigated the LPS effect on the eliciting phase of murine allergic rhinitis model. An experimental protocol of murine allergic rhinitis model is briefly described. at the eliciting phase, OVA antigens are intranasally introduced for 7 consecutive days with LPS or without LPS, and on the final challenge , sneezing rates are counted as well as nasal tissue analysis and Th2 cytokines detected with Immunoprecipitation and western-blotting.

As a result, as shown in Fig.5, LPS aggravated the eliciting phase of type-I allergic reaction, in a murine allergic rhinitis model. Furthermore, the significant difference in sneezing rates between C3H/HeN mice challenged with OVA alone and OVA with LPS was found, but this difference was not detected in C3H/HeJ mice. Eosinophil infiltration was more prominent in C3H/HeN mice challenged with OVA and LPS, in comparison with those in mice challenged with OVA alone. In western blot analysis, IL-5,IL-10,IL-13 expression was seen in both groups, but IL-5 expession was upregulated in mice challenged with OVA and LPS. However, there was no significant difference in eosinophil infiltration and Th2 cytokine expression between C3H/HeJ mice challenged with OVA alone and OVA with LPS. These data taken together suggests that LPS aggravates nasal symptom, upregulating Th2 cytokine production of mast cells via TLR4.

DISCUSSION AND FUTURE GOAL

In the present study, Toll-Like receptors expressed on epithelial cells, mast cells, and macrophages residing in upper respiratory tract mucosae, are demonstrated to have an important role on the pathogenesis of persistent inflammation in nasopharyngeal cavity and middle ear cleft. Therefore, paranasal sinus or middle ear persistent inflammation might be explained by such an interaction between bacterial degradation product and Toll-like receptors on resident epithelial cells and/or recruited inflammatory cells in there. Furthermore, innate Immunity is highly evaluated to non-specifically evacuate nasopharyngeal or middle ear pathogens via Toll-like receptors on epithelial cells and/or recruited inflammatory cells into the paranasal sinus or middle ear. To this end, our results may lead us new therapeutic strategy (H1 receptor antagonists, signal transduction inhibitors, anti-sense therapy) to down regulate the stagnant inflammation in paranasal sinuses or tubotympanum.

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ORIGINAL ARTICLE

Role of interleukin-15 in the development of mouse olfactory nerve

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ABSTRACT **Interleukin (IL)-15 interacts with components of the IL-2 receptor (R) and exhibits T cell-stimulating activity similar to that of IL-2. In addition, IL-15 is widely expressed in many cell types and tissues, including the central nervous system. We provide evidence of a novel role of IL-15 in olfactory neurogenesis. Both IL-15 and IL-15Rα were expressed in neuronal precursor cells of the developing olfactory epithelium in mice. Adult IL-15Rα knockout mice had fewer mature olfactory neurons and proliferating cells than wild-type. Our results suggest that IL-15 plays an important role in regulating cell proliferation in olfactory neurogenesis.**

Key Words: embryo, interleukin-15, knockout mouse, olfactory epithelium, olfactory neurogenesis

INTRODUCTION

Interleukin (IL)-15 is a 14- to 15-kDa member of the 4-α helix bundle family cytokines that shares biological activities and receptor components with IL-2. IL-15 and IL-2 have many overlapping functions (Burton *et al.* 1994; Grabstein *et al.* 1994; Tagaya *et al.* 1996a), but the expression and function sites of IL-2 and IL-15 differ. IL-2, but not IL-15, is produced by activated T cells, and they bind their unique respective receptor subunits, IL-2 receptor alpha (IL-2Rα) and IL-15 receptor alpha (IL-15Rα). The respective receptors also include the IL-2 receptor beta (IL-2Rβ) and IL-2 receptor gamma/gamma common (IL-2Rγ/γc) subunits (Tagaya *et al.* 1996a). IL-15Rα binds IL-15 with high affinity (Kd, 1×10^{-11} M) even in the absence of IL-2Rβ and IL-2Rγ/γc subunits. IL-15 and IL-15R mRNA are expressed in various cell types and tissues, including nerve cells and the brain (Grabstein *et al.* 1994; Tagaya *et al.* 1996b; Budagian *et al.* 2006). Both IL-15 and IL-15Rα are expressed throughout the central nervous system (CNS) of fetal mice (Hanisch *et al.* 1997) and humans (Kurowska *et al.* 2002). Beta-III tubulin-positive E15 mouse neurons showed diffuse IL-15 immunostaining in both soma and neurites, and IL-15 expression in mouse brain, including olfactory bulb (OB) and olfactory nucleus have been reported (Gómez-Nicola *et al.* 2008), while the role of IL-15 in the olfactory nervous system remains unknown. Here, we provide evidence for a novel role of IL-15 in the mouse olfactory nervous system.

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MATERIALS AND METHODS

Mice

Mice from the Institute of Cancer Research (ICR) (CLEA Japan, Tokyo) aged 10–20 weeks were mated. We defined noon of the day during which a vaginal plug was observed as embryonic day (E) 0.5. Embryos were obtained from E12.5 to E18.5. We purchased BALB/c mice (CLEA Japan). IL-15Rα knockout (IL-15Rα$^{-/-}$) mice that originated from the Tokyo University repository at Jackson Laboratories (Bar Harbor, ME, USA) were derived from over six generations of backcrossing onto the BALB/c background. IL-15Rα$^{-/-}$ and wild-type mice (five males and three females for each) were used for the following immunostaining and measurements. We used the mice at 8 or 11 weeks of age. The study protocol was approved by the Ethics Committee for Animal Experimentation of Shimane University, and the animals were handled according to the institutional guidelines.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Pregnant ICR mice were anesthetized, and the embryos were removed by cesarean section and killed under ether anesthesia. Adult ICR mice were killed by cervical dislocation after anesthesia. Total RNA was extracted from the olfactory epithelium (OE) and OB that were carefully dissected out from the embryos ($n = 10$ per each embryonic day) and adults ($n = 2$), and then the expression of *IL-15* and *IL-15Rα* mRNA was examined by RT-PCR. An IL-15 primer pair was purchased from R & D Systems (Minneapolis, MN, USA). The primers for IL-15Rα were forward, 5′-GGGGTTGTGATG GCTTTCCTGG-3′ and reverse, 5′-GTTTCCATGGTTTCCACC TCAA-3′. The predicted sizes of the amplified DNA fragments for *IL-15* and *IL-15Rα* were 266 and 86 bp, respectively. The PCR reaction proceeded over 35 cycles of 94°C for 1 min, 55°C for 1 min (for IL-15) and 58°C for 1 min (for IL-15Rα), followed by 72°C for 1 min. The amplified PCR products were resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide, and verified using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA).

Tissue preparation

Whole embryos were embedded in Tissue-Tek® Optimal Cutting Temperature compound (Sakura Finetek Japan, Tokyo, Japan) and stored at −80°C. Frozen coronal sections were cut (14 μm) and fixed with 4% paraformaldehyde at room temperature (RT) for 20 min (IL-15) or acetone at −20°C for 30 min (IL-15Rα). For adult IL-15Rα$^{-/-}$ and wild-type mice, whole head regions were fixed in 4% formaldehyde at 4°C overnight, decalcified in ethylenediaminetetraacetic acid 2Na at RT for 3 weeks and embedded in paraffin.

Immunohistochemistry

Coronal sections of the embryo head were stained with rabbit polyclonal antiIL-15 (1:500; Abcam, Cambridge, UK) and goat polyclonal antiIL-15Rα, or goat polyclonal antiIL-15, and rabbit polyclonal antiIL-15Rα (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Immature and mature olfactory receptor neurons (ORN) were, respectively, stained with monoclonal antigrowth-associated protein (GAP)-43 (1:200; Zymed Laboratories, South San Francisco, CA, USA) and goat polyclonal antiolfactory marker protein (OMP) (1:500; Wako Pure Chemical Industries, Richmond, VA, USA) antibodies. The proliferation and apoptosis of OE cells were examined using monoclonal antiproliferating cell nuclear antigen (PCNA) (1:3000; Abcam) and rabbit polyclonal antisingle-stranded DNA (ssDNA) (1:400; Dako, Carpinteria, CA, USA) antibodies.

Embryonic sections were incubated with Cy3-conjugated donkey polyclonal antimouse IgG (1:500; Chemicon, Temecula, CA, USA), polyclonal antigoat IgG (1:500; Chemicon) and biotinylated antirabbit IgG (1:200; Chemicon) after an overnight incubation at 4°C with primary antibodies. Sections were then incubated with fluorescein isothiocyanate-conjugated ExtrAvidin (Sigma, St. Louis, MO, USA) and observed using a confocal laser microscope (Zeiss, Thornwood, NY, USA).

Adult sections were incubated overnight at 4°C with antiOMP, antiPCNA, or antissDNA antibodies and microwaved before incubation with primary antibodies in 10 mM citric acid (pH 6.0) for PCNA, or digested with 20 μg/mL proteinase K at 37°C for 20 min for ssDNA. The sections were incubated with Histofine Simple Stain Mouse MAX PO (goat) (Nichirei, Tokyo, Japan), Mouse on Mouse Immunostaining Kit (Vector Laboratories, Burlingame, CA, USA), or ENVISION+ System HRP Rabbit (Dako). The chromogen was liquid diaminobenzidine (Dako). Nuclei were counterstained with hematoxylin. Three or more embryos were immunostained per each embryonic day for each primary antibody.

Measurements

We counted the total number of cells as well as OMP-positive, OMP-negative, ssDNA-positive and PCNA-positive cells within the OE in the area medial to the vertical line to the top of the roof of the nasal cavity and above the horizontal line through the tangential point of the vertical line to the OE of the nasal septum. Five sections were examined every 150 μm posterior from the section where the OB first appeared. We also measured the area of the olfactory nerve bundle in the lamina propria of the defined area. Data were statistically analyzed using Scheffé's post hoc test and $P < 0.05$ was regarded as significant.

RESULTS

Expression of IL-15 and IL-15Rα in mouse OE

We found by RT-PCR (Fig. 1a,b) that *IL-15* and *IL-15Rα* mRNA were expressed in the OE and in the interstitial region surrounding the OE at E12.5. This expression continued until E18.5, and was detectable in the adult OE and OB. Immunohistochemistry showed that IL-15 and IL-15Rα were expressed in the OE at E12.5-E18.5, although IL-15 immunostaining was weak until E14.5 (Fig. 1c–h, and data not shown). At E14.5, IL-15 staining (Fig. 1c) was detected in the axons of olfactory neurons, and was co-localized with GAP-43 (Fig. 1d), a marker of immature neurons (Van der Zee *et al.* 1989; Verhaagen *et al.* 1989) (Fig. 1e). Like IL-15 staining, IL-15Rα immunostaining was co-localized with GAP-43 in olfactory neuron axons, and was detected in the OE (Fig. 1f). At E18.5, IL-15 and OMP stainings were overlapped in axon bundles of olfactory neurons and the OE (Fig. 1g). Likewise, IL-15Rα immunostaining was co-localized with OMP (Fig. 1h). OMP is expressed almost exclusively by ORN and is considered the gold standard marker for mature ORN (Farbman and Margolis 1980; Margalit and Lancet 1993; Buiakova *et al.* 1994). During olfactory neurogenesis, among markers of developing neurons throughout the central and peripheral neurons, expression of GAP-43 is very similar to that of beta-III tubulin, while only the most mature neurons express the OMP, and OMP expression appears to be upregulated at the same time that beta-III tubulin expression is reduced (Roskams *et al.* 1998).

Comparison of OE between KO and WT mice

To understand the role of IL-15 in olfactory neurogenesis, we first compared the number of OMP-positive cells (Fig. 2h) and the area of OMP-positive olfactory nerve bundles (Fig. 2i) in the lamina propria between adult IL-15Rα$^{-/-}$ and wild-type mice (male, Fig. 2b, f vs a, e; female, Fig. 2d, g vs c). OMP-positive cells were fewer in IL-15Rα$^{-/-}$ than in wild-type mice (Fig. 2h), but no significant difference was found in OMP-negative cells (data not shown). In addition, the area of OMP-positive olfactory nerve bundles was smaller in IL-15Rα$^{-/-}$ mice than in wild-type mice (Fig. 2i). Nasal glands appeared to overdevelop in the lamina propria of IL-15Rα$^{-/-}$ mice due to the decreased development of axons (Fig. 2b,d,f,g). The basement membrane was not clearly identified, and the epithelium protruded irregularly into the lamina propria of IL-15Rα$^{-/-}$ mice (Fig. 2f,g). We next counted PCNA-positive cells in the OE (Fig. 2j), since PCNA is a marker of the early G1 and S phases of the cell cycle. Most of PCNA-positive cells are located in the basal cell layer of the OE, while some are found in the supporting cell layer (data not shown). Male IL-15Rα$^{-/-}$ mice contained significantly fewer PCNA-positive cells than male wild-type mice, whereas female IL-15Rα$^{-/-}$ and wild-type mice did not significantly differ (Fig. 2j). We further investigated apoptotic cells in the OE of IL-15Rα$^{-/-}$ and wild-type mice (Fig. 2k) by immunostaining using antisingle-stranded DNA antibody (Nimura *et al.* 2008). The number of ssDNA-positive cells did not significantly differ between IL-15Rα$^{-/-}$ and wild-type mice (Fig. 2k).

DISCUSSION

The multifunctional cytokine IL-15 was originally identified in proliferating immune cells, but it has become increasingly recognized as a major modulator of many types of non-immune cells. Many effects of IL-2 on neuronal cells have been reported, suggesting that IL-15 also functions in neuronal development (Hanisch and Quirion 1995). Hanisch *et al.* (1997) reported that IL-15 acts as an autocrine growth factor and supports microglial cell growth. The present results revealed that the number of OMP-positive, but not of OMP-negative cells, in the OE was affected by the absence of IL-15Rα suggesting that IL-15 signaling is involved in the development of olfactory sensory neurons and precursor cells.

The distribution of *IL-15* and *IL-15Rα* mRNA and proteins observed in the present study suggests that IL-15 acts as a growth factor through an autocrine or paracrine loop, and IL-15Rα deficiency appears to disrupt development of the olfactory neuron system. IL-15 is a potent inhibitor of several apoptosis pathways (Bulfone-Paus *et al.* 1997) and controls the homeostasis and growth of immune cells (Lodolce *et al.* 1998) as well as different types of non-immune cells (Budagian *et al.* 2006). However, the present results suggest that the IL-15 pathway is related to the proliferation, but not to the apoptosis of olfactory neurons in adult mice, although

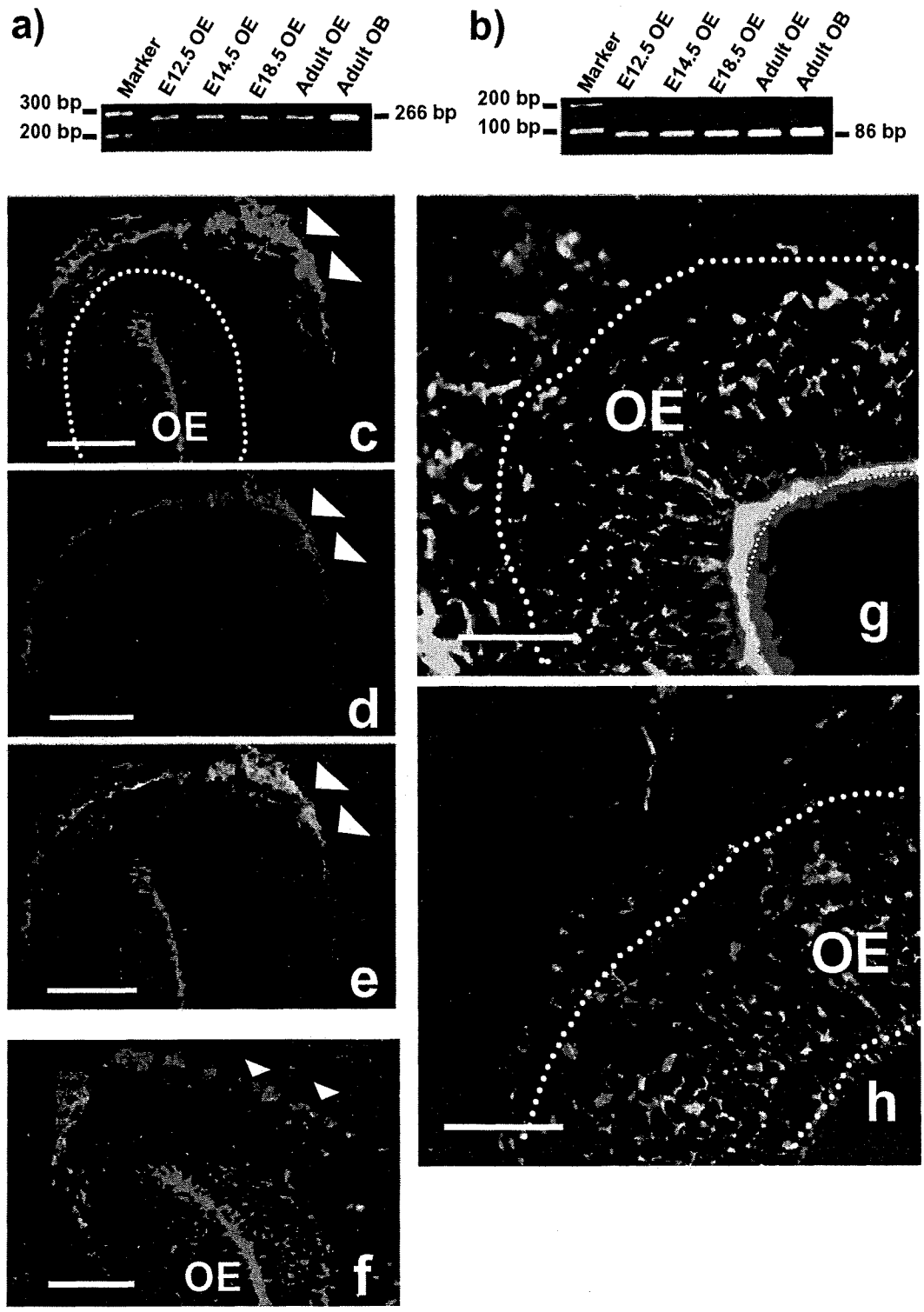

Fig. 1 Expression of interleukin (IL)-15/IL-15Rα mRNA and IL-15/IL-15Rα proteins in embryonic olfactory neurons. IL-15 (a) and IL-15Rα (b) mRNA are expressed in olfactory epithelium (OE) at E12.5 onward and in adult OE and olfactory bulb (OB). This figure shows the result of representative one from triplicated experiments. IL-15 (c, green) is immunostained in axons of olfactory neurons, in GAP-43-positive immature neurons (d, red) at embryonic day (E)14.5 (e, merged). IL-15Rα (green) is immunostained in GAP-43-positive (red) immature neurons at E14.5 in axons of olfactory neurons (f, merged), similar to IL-15. At E18.5, IL-15 (green) is immunostained in OMP-positive mature olfactory neurons (red), in the OE (g; merged). IL-15Rα (green) is immunostained in OMP-positive neurons (red) at E18.5 in OE (h; merged), similar to IL-15. Arrowheads indicate axon bundles of olfactory neurons (c–f). Bars, 100 μm (c–f), and 50 μm (g, h).

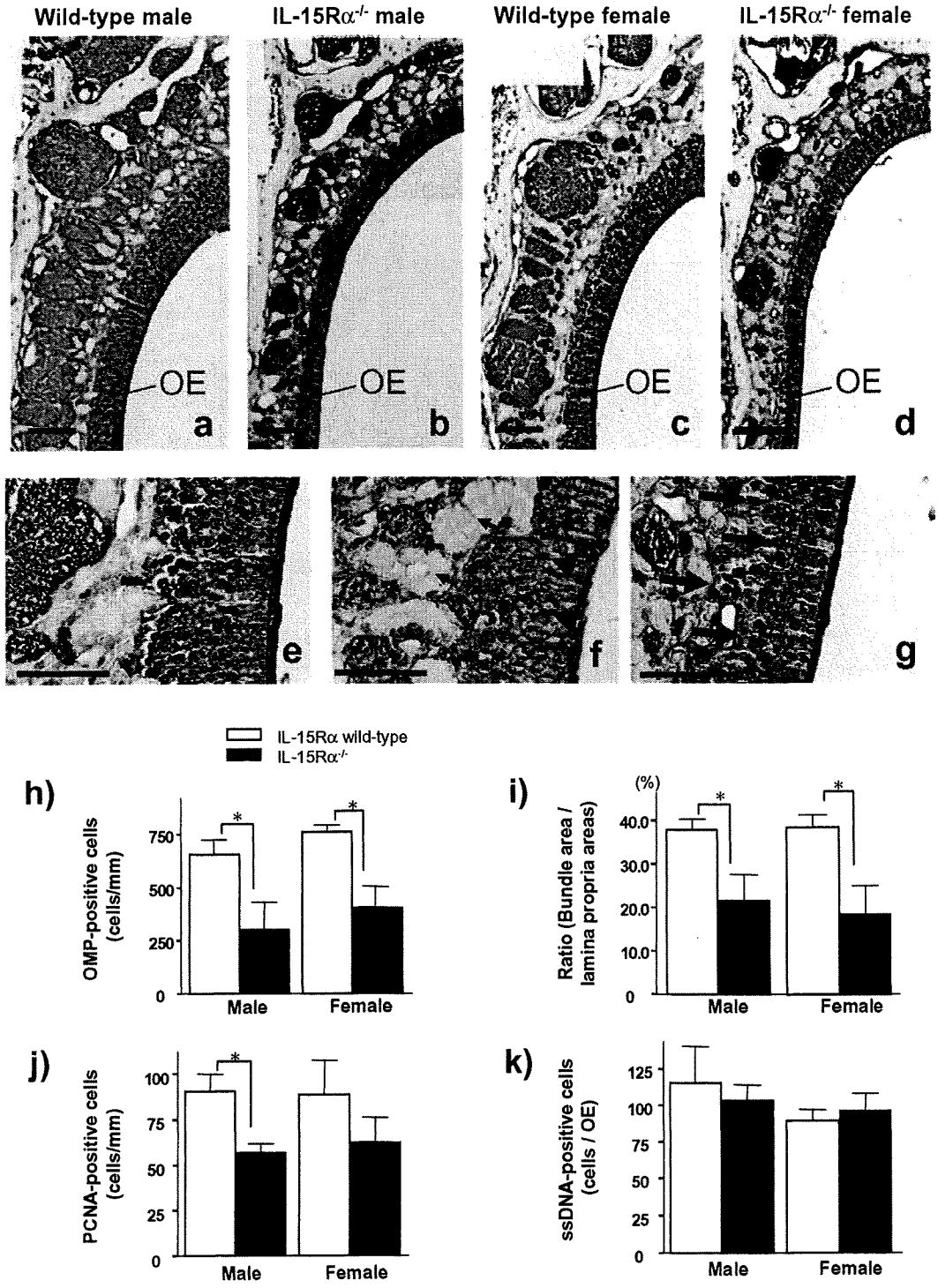

Fig. 2 Neuronal development in olfactory epithelium (OE) is inhibited by interleukin (IL)-15Rα deficiency because of decreased proliferative activity but not promoted apoptosis of OE cells. The OE is thinner in IL-15Rα$^{-/-}$ male (b, f) and female (d, g) mice than in wild-type male (a, e) and female (c) mice. (h) Numbers of OMP-positive olfactory neurons (arrowheads in f) were decreased by IL-15Rα deficiency. (i) In addition, the ratio of area of OMP-positive nerve bundles (asterisks in a–d) to that of lamina propria was also significantly reduced in IL-15Rα$^{-/-}$ (b, d) mice compared with wild-type mice (a, c). Nasal glands appeared to overdevelop in lamina propria instead of underdeveloped axons in IL-15Rα$^{-/-}$ mice (arrows in b, d, and f). Compared with wild-type mice (a, c, e), basement membrane was not clearly identified, and epithelium irregularly protruded into lamina propria in IL-15Rα$^{-/-}$ mice (large arrows in g). (j) Male IL-15Rα$^{-/-}$ mice had significantly fewer PCNA-positive cells (data not shown) than male wild-type mice, whereas the numbers did not significantly differ between female IL-15Rα$^{-/-}$ and wild-type mice. (k) The number of apoptotic, ssDNA-positive cells did not significantly differ between IL-15Rα$^{-/-}$ mice and wild-type mice. Bars, 100 μm (a–d), 50 μm (e–g).

we cannot exclude the possibility that the role of IL-15 differs between embryonic and adult stages.

Lymphopenia arises in IL-15Rα$^{-/-}$ mice, not because of apoptosis, but because of the decreased proliferation and homing of IL-15Rα$^{-/-}$ lymphocytes to peripheral lymph nodes (Lodolce *et al.* 1998), suggesting that IL-15Rα is involved in the maintenance of lymphoid homeostasis. The number of OMP-positive cells and the area of OMP-positive olfactory nerve bundles in the lamina propria in IL-15Rα$^{-/-}$ mice might have decreased because of disordered homeostasis. We found that most PCNA-positive cells, which appear to comprise neuronal precursor cells, were located in the basal layer of the OE. The number of PCNA-positive cells in male IL-15Rα$^{-/-}$ mice was significantly lower than that in male wild-type mice, but it did not differ between female IL-15Rα$^{-/-}$ and wild-type mice (Fig. 2j). Hippocampal IL-15 expression is increased in IL-2-knockout mice, and neurogenesis is increased in male, but not in female IL-2-knockout mice (Beck *et al.* 2005). This is consistent with our present results showing defective neurogenesis in male IL-15Rα$^{-/-}$ mice. The distinctive profiles of males and females suggest that compensatory mechanisms for IL-15 signaling function in female mice.

Some autoimmune and inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis (MS) (Pashenkov *et al.* 1999) and Alzheimer's disease (AD) (Rentzos *et al.* 2006) might be related to the IL-15/IL-15R system. Autoimmunity and cytokine dysregulation are apparently associated with MS (Steinman 1996), and patients with MS have olfactory dysfunction (Zivadinov *et al.* 1999). The results of the Cross-Cultural Smell Identification Test showed that average scores are lower in patients with MS than in controls. In addition, scores are significantly poorer in men than in women with MS (Zivadinov *et al.* 1999). We posit that IL-15 is involved in the olfactory dysfunction of MS and that compensatory mechanisms exist in women. Defective IL-15 signaling might thus result in diseases characterized by olfactory dysfunction, especially in men. AD is a neurodegenerative disorder that results in a major cognitive decline and it is associated with low-grade but sustained inflammation and elevated IL-15 in the cerebrospinal fluid (Rentzos *et al.* 2006). Pathological changes involve both sensory and non-sensory areas of the OE in patients with AD (Talamo *et al.* 1989), and are consistent with the present findings of OMP immunostaining in male IL-15Rα$^{-/-}$ mice. Thus, IL-15 is an important factor for olfactory neurogenesis and it might be related with the pathogenesis of neurological disorders involving cytokine dysregulation.

CONCLUSION

The present results have demonstrated the evidence for a role of IL-15 in olfactory neurogenesis. In the developing olfactory epithelium of mice, IL-15 and IL-15Rα were expressed in neuronal precursor cells by RT-PCR and immunohistochemistry. In adult mice, numbers of mature olfactory neurons, but not non-neuronal cells, in the olfactory epithelium were less in IL-15Rα$^{-/-}$ mice than in wild-type mice. Numbers of proliferating cells in male, but not female, IL-15Rα$^{-/-}$ mice were less than those of wild-type mice, whereas the numbers of apoptotic cells did not differ between IL-15Rα$^{-/-}$ and wild-type mice. These results suggest that IL-15 plays an important role in regulating neuronal proliferation during olfactory neurogenesis.

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