It is well known that peptidoglycan, LTA, lipoproteins and genomic DNA from Gram-positive bacteria, including lactobacilli, stimulate TLR2 and TLR9, leading to the secretion of proinflammatory cytokines.[28] However, other published reports have revealed that TLR2- or TLR9-deficient as well as wild-type macrophages respond to certain *Lactobacillus* strains that belong to the *L. casei* group and secrete IL-12.[13,29] In this study, we reconfirmed that *L. casei* induced a high level of IL-12 production in both wild-type and TLR2-deficient macrophages, while peptidoglycan induced a low level of IL-12 production in wild-type macrophages and even lower levels in TLR2-deficient macrophages (Fig. 9). TLR2 may be responsible for the lower level of IL-12 production induced by peptidoglycan or *Lactobacillus* strains sensitive to intracellular digestion, while some unidentified receptors other than TLRs may play an important role in recognizing the three-dimensional structure of the cell wall of *Lactobacillus* strains resistant to intracellular digestion, leading to the production of a higher amount of IL-12. Receptors that are strongly associated with the phagocytosis of bacteria, such as C-type lectin receptors, might be candidate receptors of the second type.[30]

Zeuthen *et al.*[12] reported that *Lactobacillus reuteri* and *Bifidobacterium bifidum*, which are weak inducers of IL-12, inhibited the *Lactobacillus acidophilus*-induced potent production of IL-12 by human dendritic cells in an IL-10-independent manner. They further revealed that the *B. bifidum*-induced inhibition of IL-12 production was mediated by the recognition of lipoproteins and partially ruptured insoluble cell walls by TLR2 on murine dendritic cells, and they suggested that TLR2-mediated signals could act as negative regulators of proinflammatory responses.[31] Our findings showed that *L. johnsonii* and *L. plantarum*, which were weak inducers of IL-12, inhibited IL-12 production by involving the TLR2-mediated signalling pathway and they agree well with the results of Zeuthen *et al.* However, our results showed that peptidoglycan was responsible for the inhibitory effect of *L. johnsonii* and *L. plantarum*, mainly through its recognition by TLR2; in contrast, Zeuthen *et al.* proposed the possibility that peptidoglycan promotes IL-12 production via the recognition of its digestion products by NOD2. These contradictory findings may be explained by the different responses elicited by dendritic cells and macrophages, as other researchers have also revealed a difference in responses of these cells: NOD2 mediated synergistic induction of IL-12 production in dendritic cells, while it was found to mediate the inhibition of IL-12 production in macrophages.[9,32] The cross-regulatory effect of certain probiotics has also been observed in an *in vivo* study, wherein it was found that the anti-allergic effect of a mixture of *L. rhamnosus* GG and three other probiotic strains was weaker than that elicited by the *L. rhamnosus* GG strain alone in the treatment of infant atopic dermatitis.[33] These findings suggest that these cross-regulatory effects should be considered when developing novel probiotic mixtures.

Probiotics having a potent IL-12-inducing ability are expected to exhibit suppressive effects on infections and cancers, as IL-12 induces a Th1-dominant immune response and augments the innate immune response. However, some researchers are afraid that certain probiotics, which induce excessive production of IL-12, may increase the risk of inflammatory diseases.[34] Our present finding that easily digestible lactobacilli and peptidoglycan inhibit the IL-12 production potently induced by a probiotic *L. casei* strain supports the idea that, when orally administered, such probiotics may stimulate mucosal macrophages simultaneously with intestinal commensal lactobacilli or their peptidoglycan to produce suitable levels of IL-12, and thereby have beneficial rather than adverse effects on the host.

Intestinal macrophages and dendritic cells have been reported to secrete only low levels of proinflammatory cytokines such as IL-12 in response to bacterial stimuli, and this may contribute to the maintenance of intestinal homeostasis. The suppressed responses of intestinal macrophages and dendritic cells are thought to be mediated by various mechanisms, such as the constitutive expression of inhibitory nuclear transcription factors in these cells and the supply of suppressive mediators by intestinal epithelial cells.[27,35,36] Our study showed that peptidoglycans from both *S. aureus* and lactobacilli had an inhibitory effect on IL-12 production, suggesting that peptidoglycans from many bacteria in addition to lactobacilli might inhibit the IL-12 production potently induced by certain other bacteria. The intestine hosts various commensal bacteria, and some of them may be able to inhibit IL-12 production through recognition of peptidoglycan and its digestion products via TLR2 and NOD2. This inhibitory mechanism may also contribute to the maintenance of intestinal homeostasis.

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Disclosures

The authors have no conflict of interest.

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EDITOR LUISA BELLUSSI

LIBER AMICORUM

RAPHAEL'S SCHOOL *of* ATHENS

Hideyuki Kawauchi, MD, DMSc

Curriculum vitae

Biographical Information

Born: August 19, 1954, Nagasaki, JAPAN
Marital Status: Married, Three children

Academic Background

1973-M.D. Yamaguchi University, School of Medicine - Yamaguchi, JAPAN

1979-D.MSc Postgraduate Course, Department of Immunology, Institute of Bioregulation Research, Kyushu University - Fukuoka, JAPAN

Professional Background

1984-1984 Assistant, Department of Otolaryngology - Oita Medical University, Oita, JAPAN (prof. Goro Mogi)

1994-1994 Assistant Professor, Department of Otolaryngology - Oita Medical University, Oita, JAPAN

1994-present Professor and Chairman, Department of Otolaryngology, Faculty of Medicine, Shimane University, Shimane JAPAN

Oversea Research Experience

1986-1988 Research Fellow, Otological Research Laboratory - Department of Otorhinolaryngology, The Ohio State University, School of Medicine, USA September 1986-March 1988 (prof. David J Lim)

Main Past and Present Administrative Responsibilities in ENT field

1993 Symposium coordinator, 2nd Extraordinary International Symposium on Recent Advances in Ototis Media, Oita, Japan, March 31-April 3.

1994 Symposium coordinator, 4th International Academic Conference on Immunology in Otology, Rhinology and Laryngology, Oita, Japan, April 4-7.

2002 President at 20th annual meeting, The Japan Society of Immunology & Allergology in Otolaryngology, in Shimane, Japan, March 7-9.

2007 President at 17th annual meeting, The Japan Society of Head and Neck Surgery, in Shimane, Japan, February 1-2, 2007.

2008 President at 38th annual meeting, Japan Society of infectious disease in Otolaryngology, and 32nd annual meeting, Japan Society for Earosols in Medicine, in Shimane, Japan, September 5-6, 2008.

2009 President at 48th annual meeting, Japan Rhinologic Society, in Shimane, Japan, October 1-3,2009.

Membership for international society

Politzer Society, Association for Research in Otolaryngology (USA), European Rhinologic Society, Society for Mucosal Immunology

Editorial activity

Editor in chief, Japanese Journal of Otorhinolaryngological Society (2006-present)

Review Board Member at present

Rhinology, Acta Oto-Laryngologica, Laryngoscope, Auris Nasus Larynx, International Immunopharmacology

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

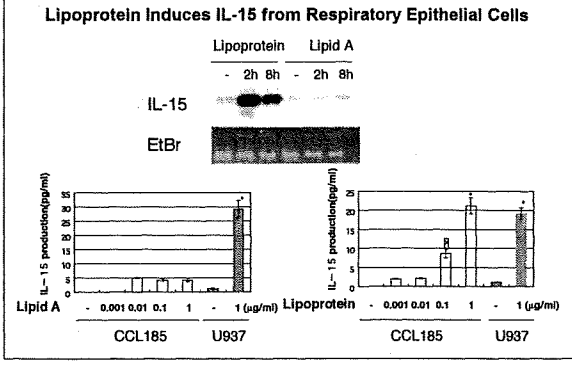


Figure 2

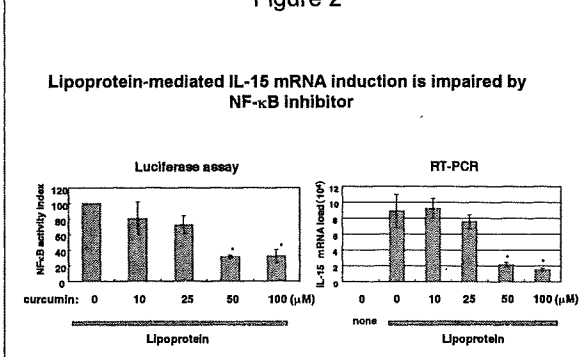


Figure 3

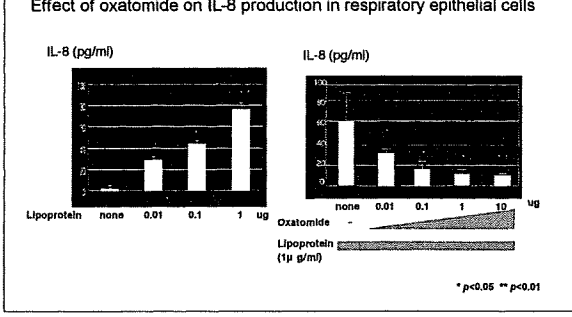


Figure 4

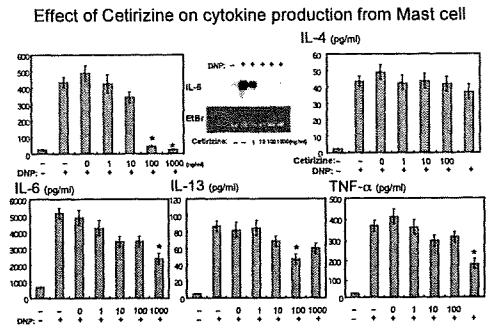
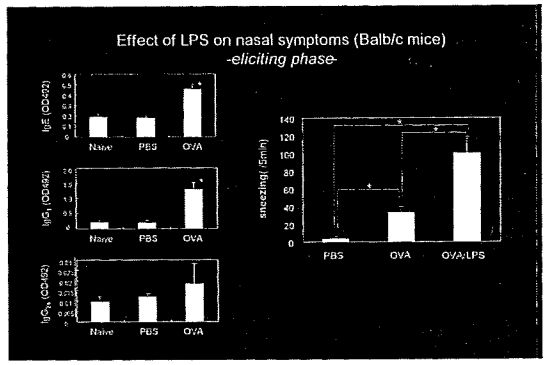


Figure 5



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

Tsuyoshi Umehara[1], Jun Udagawa[2], Kaoru Takamura[1], Mitsuhiro Kimura[1], Ryotaro Ishimitsu[1], Hiroshi Kiyono[3], Hideyuki Kawauchi[1], and Hiroki Otani[2]

Departments of [1]Otorhinolaryngology and [2]Developmental Biology, Faculty of Medicine, Shimane University, Izumo, and [3]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan

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.

Correspondence: Tsuyoshi Umehara, MD, Department of Otorhinolaryngology, Faculty of Medicine, Shimane University, Izumo 693-8501, Japan. Email: umehara@med.shimane-u.ac.jp

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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

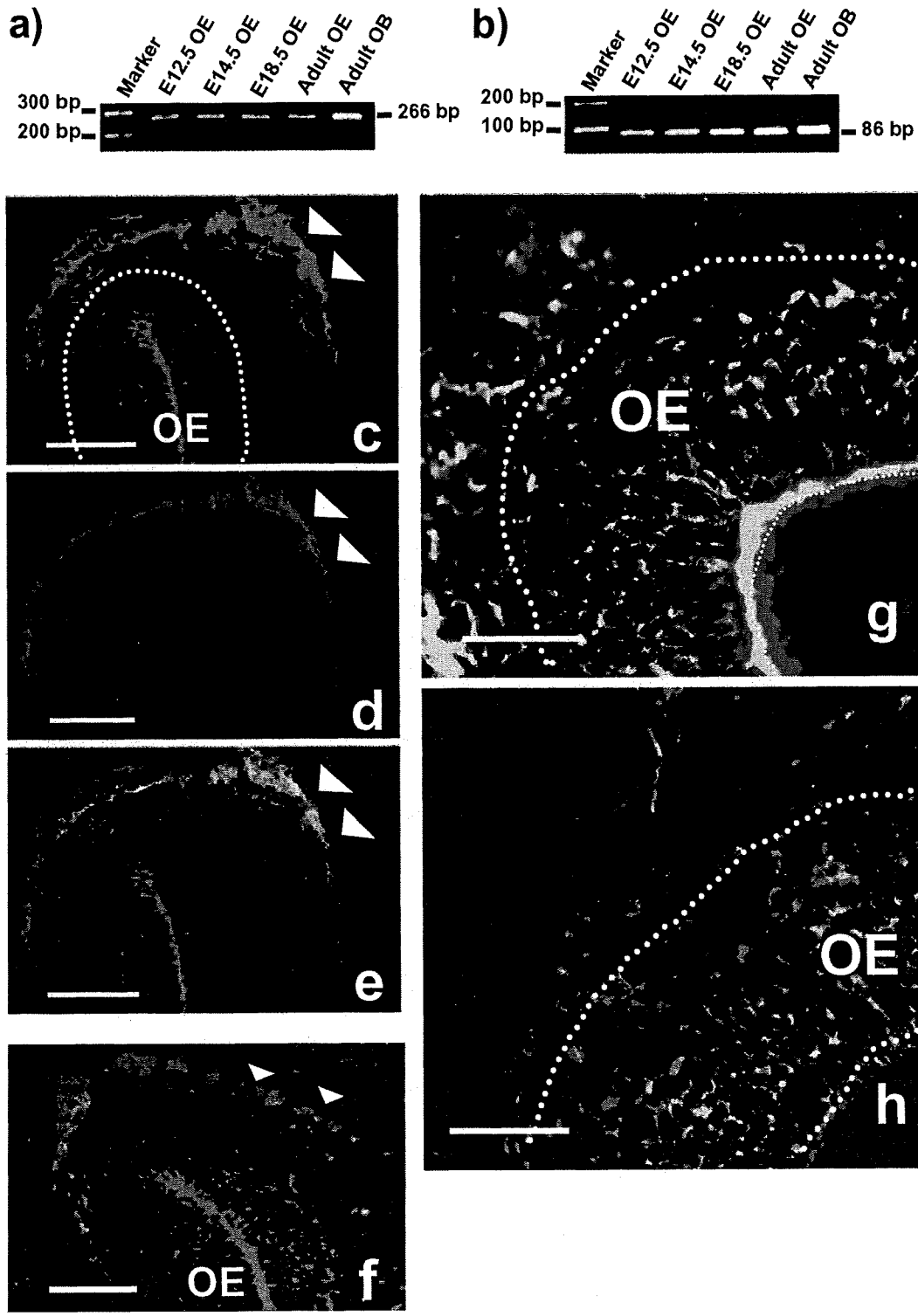

Fig. 1 Expression of interleukin (IL)-15/IL-15Rα mRNA and IL-15/IL-15Rα proteins in embryonic olfactory neurons. IL-15 (a) and IL-15Rα (b) mRNA are expressed in olfactory epithelium (OE) at E12.5 onward and in adult OE and olfactory bulb (OB). This figure shows the result of representative one from triplicated 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).

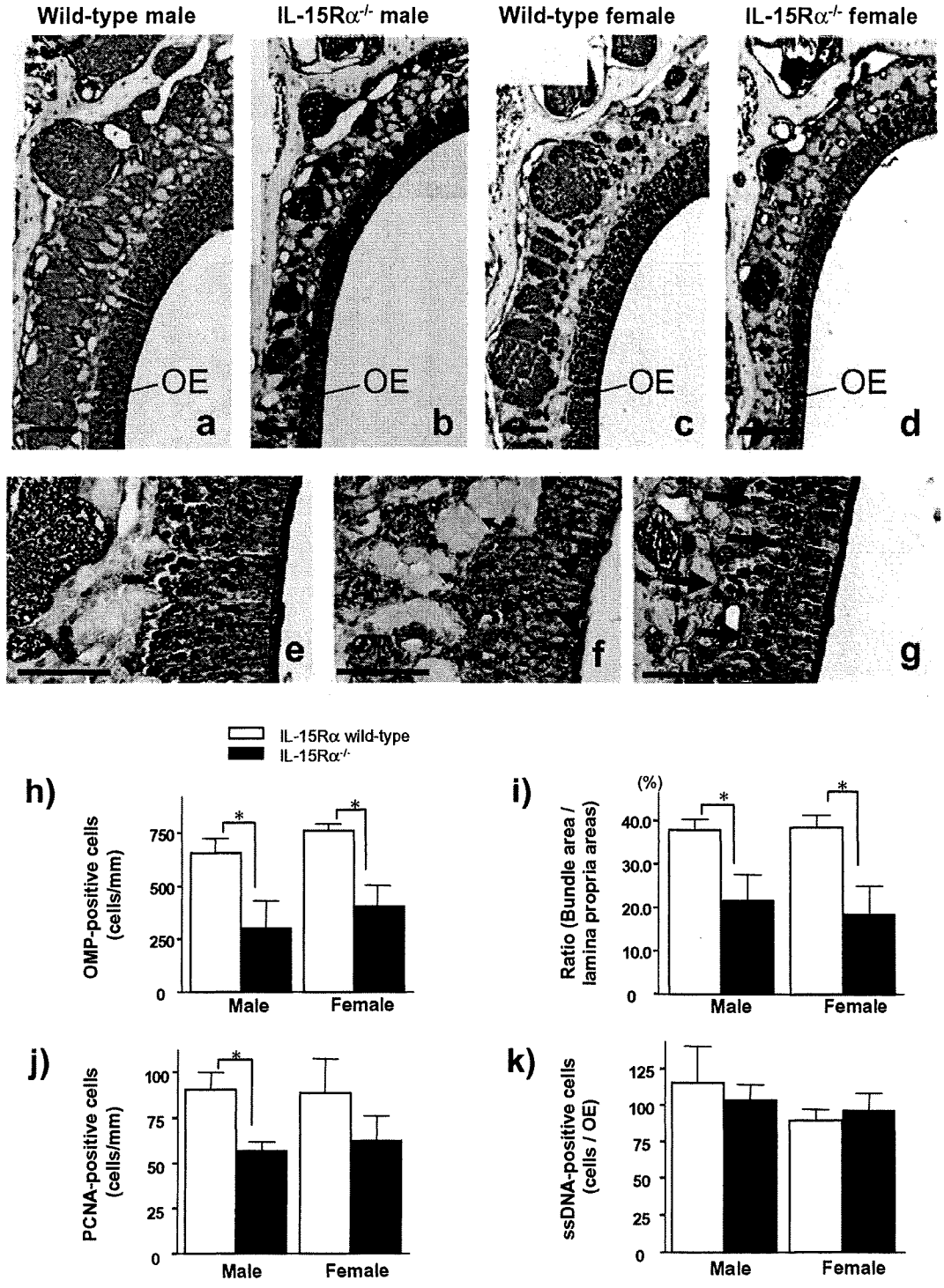

Fig. 2 Neuronal development in olfactory epithelium (OE) is inhibited by interleukin (IL)-15Rα deficiency because of decreased proliferative activity but not promoted apoptosis of OE cells. The OE is thinner in IL-15Rα$^{-/-}$ male (b, f) and female (d, g) mice than in wild-type male (a, e) and female (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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