of AID-expressing cells in both TALT and NALT (Fig. 6, G, and H). Furthermore, RT-PCR analysis confirmed *Aid* expression in TALT and NALT but not in the nasal passage (NP; Fig. 6 I). Therefore, after ocular immunization with CT, increased numbers of IgA$^+$B220$^-$ plasma cells were detected in the diffuse region of the tear duct (Fig. 6, J and K). An ELISPOT assay confirmed that some of these IgA$^+$B220$^-$ plasma cells produced CT-specific IgA; cells producing IgA specific for the B subunit of CT (CT-B), but not IgG-forming cells, were found in single-cell preparations from the tear ducts of mice ocularly immunized with CT (Fig. 6 L and not depicted). In addition, the production of CT-B–specific IgG-forming cells was induced in the spleen by ocular immunization with CT (Fig. 6 M). Naive mice did not show any CT-B–specific Ig-producing cells (Fig. 6 N).

In addition, we found a high frequency of CT-B–specific CD4$^+$ T cells in TALT using an MHC tetramer, consistent with the induction of antigen-specific antibody-producing

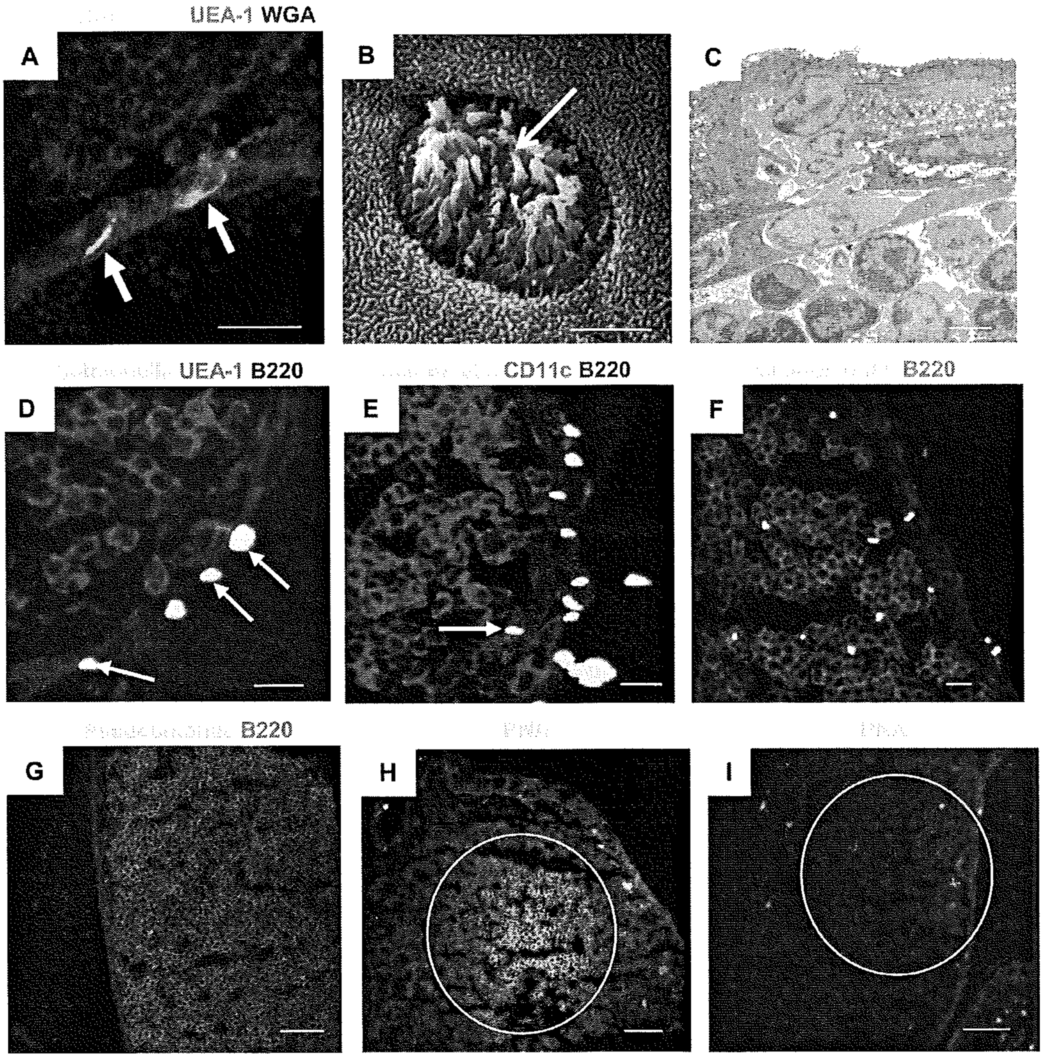


Figure 5. TALT is the site of ocular antigen uptake. (A) A confocal micrograph of TALT shows the presence of NKM16-2-4$^+$ UEA-1$^+$ M cells (arrows; $n = 3$ mice). Bar, 20 µm. (B and C) TALT-FAE was analyzed by scanning electron microscopy (B) and transmission electron microscopy (C). White and red arrows indicate M cells with the unique characteristics of microvilli and pocket lymphocytes, respectively ($n = 5$ mice/group). Bars, 3 µm. (D and E) Mice were given GFP-expressing *Salmonella* by eye drops. After 30 min, TALT was isolated and examined with confocal microscopy. Arrows in D and E point, respectively, to *Salmonella* captured by UEA-1$^+$ M cells and CD11c$^+$ DCs in TALT ($n = 3$ mice/group). Bars, 10 µm. (F) Mice were given *P. aeruginosa* PAO-1 by eye drops. After 30 min, TALT was isolated and examined with confocal microscopy. A large number of *P. aeruginosa* PAO-1 were found inside the TALT ($n = 3$ mice). Bar, 10 µm. (G) As a negative control for F, TALT from mice given PBS by eye drops were analyzed ($n = 3$ mice). Bar, 50 µm. (H) Mice were given *P. aeruginosa* PAO-1 by eye drops twice at an interval of 1 wk. 1 wk after the second administration, TALT was isolated and examined with confocal microscopy. GC formation was induced by ocular administration of *P. aeruginosa* PAO-1 ($n = 3$ mice). Bar, 50 µm. (I) TALT from a control naive mouse is shown. GCs did not form in naive TALT. These data are representative of at least two independent experiments per group ($n = 3$ mice). Bar, 50 µm.

cells after CT immunization (Fig. 7 A). It is important to note that the CT-B tetramer–reactive CD4$^+$ T cells included CXCR5$^+$ T follicular helper cells (Fig. 7 B). CT-B–specific CD4$^+$ T cells were detected in NALT and draining LNs, such as the cervical and submandibular LNs, but at a lower frequency than in TALT (Fig. 7, A and B). Thus, it is plausible

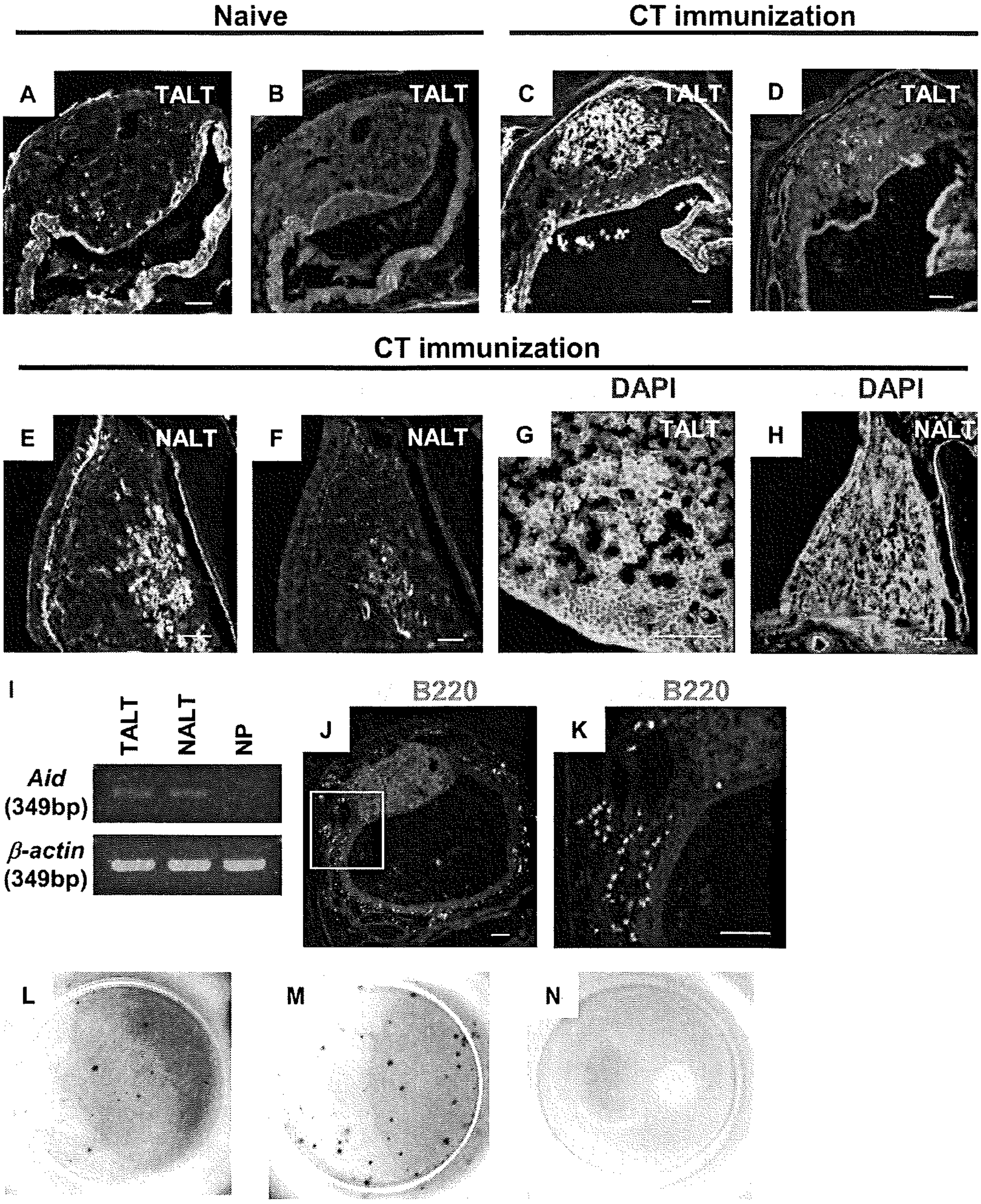


Figure 6. Induction of antigen-specific IgA responses through TALT. (A and B) Naive mice do not form GCs (n = 3 mice). (C and D) Mice were given CT by eye drops three times at 1-wk intervals. 1 wk after the last administration, TALT was isolated and examined with confocal microscopy. The CT challenge induced GC formation in TALT (C and D) and NALT (E and F; n = 3 mice/group). Tissues were stained with PNA (A, C, and E) or for FDCs (B, D, and F). (G and H) AID expression in TALT (G) and NALT (H) was detected in mice ocularly immunized with CT (n = 3 mice/group). (I) RT-PCR analysis of *Aid* expression in TALT, NALT, and NPs of mice given ocular CT (n = 3 mice/group). (J and K) Distribution of IgA$^+$B220$^-$ plasma cells in tear ducts was examined with confocal microscopy. K is a magnified view of the box in J. CT challenge by eye drops induced the appearance of a large number of plasma cells in the tear duct compartment (n = 3 mice/group). (L-N) ELISPOT analysis for the detection of CT-B-specific IgA-producing cells in tear ducts (L) and CT-B-specific IgG-producing cells in spleens (M). Data obtained from control naive mice are shown in N. These data are representative of at least two independent experiments (n = 4 mice/group). Bars, 50 µm.

that TALT is the main gateway and inductive site for the initiation of antigen-specific T and B cell responses against ocularly encountered antigens. Collectively, these observations indicate that TALT is an important member of the MALT family. It has a lymphoid structure that is organized as an inductive site for antigen uptake and the initiation of antigen-specific mucosal immune responses with GC formation and Ig class switching, as well as the generation of antigen-specific CD4$^+$ T cells.

DISCUSSION

Our purpose was to investigate the developmental features of TALT and to reveal the immunological importance of this tissue in immune surveillance for mucosal immunity. We found that the molecular requirements of TALT organogenesis were quite different from those of other secondary lymphoid organs. For example, the initiation of TALT organogenesis is independent of the IL-7R– and LTβR–NIK–mediated tissue genesis pathways, and thus, its structure was preserved in mice lacking other secondary lymphoid organs, such as *Il-7rα*$^{-/-}$, *Ltα*$^{-/-}$, and *aly/aly* mice (Table I). Furthermore, the TNF-related activation-induced cytokine (TRANCE)–mediated pathway, which is involved in pLN development (Kong et al., 1999), was dispensable for TALT genesis. Thus, the TALT structure was found in *Trance*$^{-/-}$ mice and in mice null for its signal transducer, TNF receptor–associated factor (TRAF) 6 (Fig. S5). However, these mice lacking secondary lymphoid organs (e.g., *Il-7rα*$^{-/-}$, *Ltα*$^{-/-}$, and *aly/aly* mice) had smaller TALT volumes than were found in WT mice. LTβR–NIK–mediated signals induce the production of lymphoid chemokines such as CXCL13, CCL19, and CCL21, and adhesion molecules, including VCAM-1 and PNAd (Dejardin et al., 2002; Browning et al., 2005). The immature formation of TALT in mice deficient in LTβR-associated molecules can therefore be explained by the lack of lymphoid chemokines and adhesion molecules involved in leukocyte migration. In support of this, the extent of the TALT in *Cxcl13*$^{-/-}$*plt/plt* mice was smaller than that in *Cxcl13*$^{-/-}$ and *plt/plt* single- or double-mutant mice, as well as in WT mice. However, accumulation of some B lymphocytes was seen in these mutant mice (Fig. S3). Thus, we cannot eliminate the possibility that the migration stage of B lymphocytes may also operate independently of the LTβR–NIK pathway for TALT genesis.

One of the important findings of our study is that TALT genesis occurs in both *Id2*$^{-/-}$ and *Rorγt*$^{-/-}$ mice. TALT genesis takes place normally in *Rorγt*$^{-/-}$ mice despite the fact that CD3$^-$CD4$^+$CD45$^+$ LTi cells, and as a consequence PPs and pLNs, are totally absent in these mice (Sun et al., 2000; Eberl et al., 2004). In addition, Id2 is the key transcriptional regulator for the induction and differentiation of CD3$^-$CD4$^+$CD45$^+$ LTi cells (Yokota et al., 1999). *Id2*$^{-/-}$ mice do not develop any form of secondary lymphoid tissues, including pLNs, PPs, or NALT (Yokota et al., 1999; Fukuyama et al., 2002). However, TALT development is Id2 and RORγt independent (Table I). We still found that CD3$^-$CD4$^+$CD45$^+$ cells, which we hypothesize to be TALT inducer cells, existed at TALT anlagen in both *Id2*$^{-/-}$ and *Rorγt*$^{-/-}$ mice, and we revealed that CD3$^-$CD4$^+$CD45$^+$ cells isolated from TALT anlagen did not express either *Id2* or *Rorγt*. A recent study showed that omental milky spots developed in the absence of LTi cells (Rangel-Moreno et al., 2009). Omental milky spots were found in *Id2*$^{-/-}$ and *Rorγt*$^{-/-}$ mice. However, this tissue development required the lymphoid chemokine CXCL13. Because the initiation of TALT development is independent of CXCL13, omental milky spots and TALT use different tissue

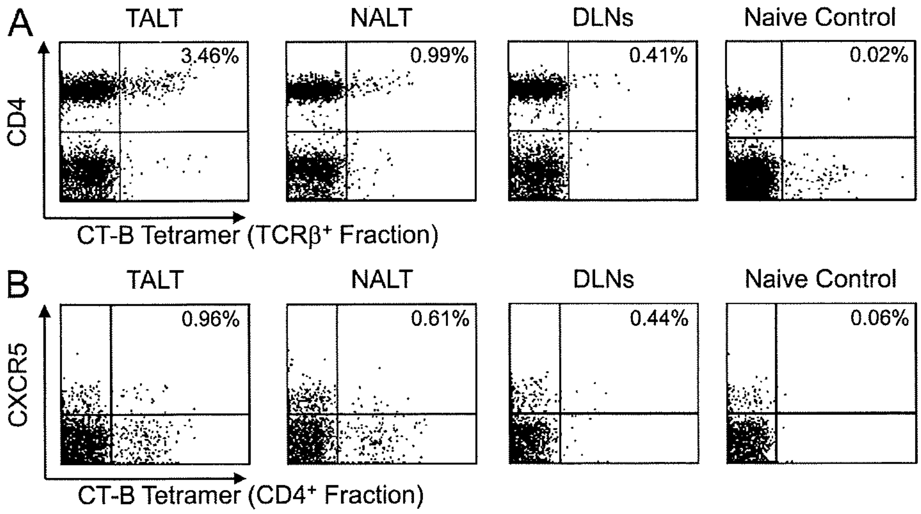


Figure 7. Induction of antigen-specific CD4$^+$ T cell responses in TALT after ocular immunization. (A and B) FACS analysis of CT-B tetramer-positive cells in lymphocytes isolated from TALT, NALT, and draining LNs (DLNs; cervical and submandibular LNs) of CT-immunized or naive mice. Data from TCRβ$^+$ (A) and CD4$^+$ (B) populations are shown. TALT preferentially responded to ocular administration of CT and generated CT-B–specific CD4$^+$ T cells, including CXCR5$^+$ T follicular helper cells. These data are representative of at least two independent experiments (n = 4 mice/group).

Table I. Distinct molecular features for organogenesis of different MALTs

Mice	TALT	NALT	PP	CLN	MLN	ILF	Cryptopatch	References
Id2$^{-/-}$	+++	–	–	–	–	ND	ND	*1
Rorγt$^{-/-}$	+++	+++	–	–	–	–	+/–	*2
Ltα$^{-/-}$	+	+	–	–	+/–	–	+/–	*3
aly/aly	+	+	–	–	–	–	++	*4
Il-7rα$^{-/-}$	++	++	–	+/–	++	++	–	*5
Cxcl13$^{-/-}$	++	+	+/–	+/–	++	ND	ND	*6
plt/plt	+++	++	++	++	++	ND	ND	*7
Cxcl13$^{-/-}$*plt/plt*	+	+	+/–	–	++	ND	ND	*8

CLN, cervical LN; MLN, mesenteric LN. +++, developed well; ++, developed with decreased number of lymphocytes; +, developed with few number of lymphocytes; –, absent; +/–, present or absent, depends on individual. *1, Yokota et al., 1999; Fukuyama et al., 2002; Boos et al., 2007; *2, Sun et al., 2000; Harmsen et al., 2002; Eberl and Littman, 2004; Eberl et al., 2004; Naito et al., 2008; Tsuji et al., 2008; *3, De Togni et al., 1994; Banks et al., 1995; Suzuki et al., 2000; Fukuyama et al., 2002; Hamada et al., 2002; Harmsen et al., 2002; Taylor et al., 2004; *4, Kanamori et al., 1996; Shinkura et al., 1999; Fukuyama et al., 2002; Hamada et al., 2002; *5, Peschon et al., 1994; Kanamori et al., 1996; Adachi et al., 1998b; Fukuyama et al., 2002; Hamada et al., 2002; Luther et al., 2003; *6, Ansel et al., 2000; Rangel-Moreno et al., 2005; Fukuyama et al., 2006; *7, Nakano et al., 1997; Rangel-Moreno et al., 2005; Fukuyama et al., 2006; and *8, Luther et al., 2003; Rangel-Moreno et al., 2005; Fukuyama et al., 2006.

genesis mechanisms. The organogenesis of secondary lymphoid tissues has been shown to require several processes, including the trafficking/accumulation of LTi cells, the differentiation/activation of specialized stromal cells, and the trafficking/accumulation of conventional lymphocytes (Mebius, 2003). In this light, the genesis of these tissues can be separated into at least two phases, initiation and maturation; in other words, the migration of LTi cells and lymphocytes, respectively, to the tissue development site. Our results indicate that the initiation of TALT genesis operates independently of the requirement for the classical tissue genesis–associated signaling cascade of IL-7R/LTβR–NIK because leukocytes, including B lymphocyte, already migrated to TALT without this pathway. Further, the unique CD3$^-$CD4$^+$CD45$^+$ cells develop without a requirement for the LTi cell–associated transcriptional regulators Id2 and RORγt, and are identified as the first hematopoietic cell population that migrates to the TALT anlagen. To directly address the critical role of Id2- and RORγt-independent CD3$^-$CD4$^+$CD45$^+$ cells (or TALT inducer cells) in the initiation of TALT genesis, our efforts are now directed toward finding and/or developing TALT-deficient mice for the necessary adoptive transfer experiment.

TALT organogenesis occurs after birth, as does NALT genesis (Fukuyama et al., 2002). In contrast, PPs and pLNs are initially generated during the embryonic period (Mebius, 2003). These findings suggest that secondary lymphoid tissue genesis can be chronologically separated into two categories: a prenatal group (PPs and pLNs) and a postnatal group (TALT and NALT). However, initiation of genesis of all of these tissues, including TALT, occurs independently of microbial stimuli.

Ocular surface antigens are taken up by NALT, and NALT might function as an inductive site for tear IgA production (Ridley Lathers et al., 1998). However, our findings suggest that TALT is a key inductive tissue for immune responses, because TALT is a more important site for the generation of antigen-specific T cells than NALT and, thus, contributes to mucosal immune responses against ocularly encountered antigens. In support of this suggestion, our study showed the presence of a mucosal gateway population of M cells in TALT that is capable of taking up ocularly administered bacterial antigens (e.g., *Salmonella*). Ocular infection with *P. aeruginosa* causes corneal ulcers and sometimes loss of vision (Liesegang, 1998), and we found *P. aeruginosa* given by ocular challenge within TALT, leading to the subsequent formation of GCs. These findings indicate that TALT plays an important role in ocular immune surveillance and protection, providing the first line of defense of the host's eyesight; we can therefore expect it to be equivalent in its capacity for immunosurveillance to the other well-known mucosal inductive tissues in the aerodigestive tract, NALT and PPs.

The lacrimal glands are effector sites for IgA production because their tissue contains large numbers of IgA-producing cells (Sullivan and Allansmith, 1984; Peppard and Montgomery, 1987; Saitoh-Inagawa, 2000). We also found that a large number of IgA$^+$B220$^-$ plasma cells were distributed around the diffuse tissues of the NPs and in the tear duct in response to CT immunization via eye drops. Thus, TALT and various tissues of the tear duct are responsible for ocular immunity as inductive and effector sites, respectively.

In summary, our results demonstrated the presence of mouse TALT, providing the first definitive evidence for the existence of Id2-, RORγt-, and LTβR-independent lymphoid tissue genesis. In addition, TALT was shown to play an important role in the induction of antigen-specific immune responses and to function in immune surveillance in ocular immunity.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were purchased from Japan SLC; germ-free and *aly/aly* mice were purchased from CLEA Japan; and *Ltα*$^{-/-}$, *Igh6*$^{-/-}$, *Tcrβ*$^{-/-}$, and *Tcrδ*$^{-/-}$ mice were purchased from the Jackson Laboratory. *Il-7rα*$^{-/-}$ mice were provided by Immunex Corp., and were also purchased from the Jackson Laboratory. *Id2*$^{-/-}$, *Rorγt*$^{-/-}$, *Cxcl13*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *MyD88*$^{-/-}$, *Cxcl13*$^{-/-}$*plt/plt*, *Trance*$^{-/-}$, and *Traf6*$^{-/-}$ mice were generated as previously described (Adachi et al., 1998a; Hoshino et al., 1999; Kong et al., 1999; Naito et al., 1999; Takeuchi et al., 1999;

Yokota et al., 1999; Kurebayashi et al., 2000; Ebisuno et al., 2003; Fukuyama et al., 2006). Animal experiments were conducted in accordance with the guidelines of and with permission provided by the Animal Care and Use Committee of the University of Tokyo.

Histological analysis. Histological analysis was performed as previously described (Fukuyama et al., 2002). The antibodies and lectins used for confocal microscopy analysis were as follows: FITC– or PE–anti-CD11c (HL3; BD), FITC–anti-CD3ε (145-2C11; BD), FITC–anti-IgA (C10-3; BD), PE–anti-B220 (RA3-6B2; BD), PE–anti-CD45 (30-F11; BD), PE– or APC–anti-CD4 (RM4-5; BD), FITC–anti-TCRβ (H57–597; BD), PE–anti-CXCR5 (2G8; BD), rabbit polyclonal anti-AID (H-80; Santa Cruz Biotechnology, Inc.), purified anti-FDC (FDC-M1; BD), purified anti-PNAd (MECA 79; BD), biotinylated anti–MAdCAM-1 (MECA-89; BD), biotinylated anti–VCAM-1 (429; BD), biotinylated peanut agglutinin (PNA; Vector Laboratories), rhodamine–UEA-1 (Vector Laboratories), Alexa Fluor 633–wheat germ agglutinin (Invitrogen), and FITC–NKM16-2-4 (Nochi et al., 2007). To visualize AID, FDC, MAdCAM-1, VCAM-1, PNAd, and PNA, FITC–anti-rabbit IgG (Santa Cruz Biotechnology, Inc.), FITC–anti–rat Ig κ chain (MRK-1; BD), FITC–anti–rat IgM (G53-238; BD), streptavidin-FITC (BD), and streptavidin-PE (eBioscience) were used as secondary antibodies or reagents. In some experiments, tissues were counterstained with DAPI (Sigma-Aldrich) to visualize the nucleus. *P. aeruginosa* PAO1 was detected with a rabbit polyclonal antibody specific for the bacterium (Abcam), followed by staining with FITC–anti–rabbit IgG (Santa Cruz Biotechnology, Inc.).

Ocular administration of bacteria. GFP-*Salmonella* (Jang et al., 2004) and *P. aeruginosa* PAO1 (Parks and Hobden, 2005) were administered as ocular antigens. After a 30-min administration of GFP-*Salmonella*, the mouse's ocular surface was washed with 100 µg/ml gentamycin. *P. aeruginosa* PAO1 is characterized by motility, biofilm formation, acyl-homoserine lactone production, and virulence in a mouse infection model (Parks and Hobden, 2005). These bacteria were cultured in Luria broth medium at 37°C for 18 h and used for ocular administration, as previously described (Jang et al., 2004). PAO1 was given twice with an interval of 1 wk (Hazlett et al., 2001).

Immunization and analysis of antigen-specific immune responses. Mice were ocularly immunized with 1 µg CT per eye (Sigma-Aldrich) in 5 µl PBS by eye drops three times at weekly intervals. Tissues or cells were collected from the heads of the ocularly immunized mice 7 d after the final immunization. To characterize CT-specific antibody responses, a CT-B–specific ELISPOT assay was used. In brief, 96-well plates (MultiScreen; Millipore) were coated with 2 µg/ml CT-B in 100 µl PBS (pH 7.4) per well for 16 h. The plates were washed three times with PBS and blocked with 100 µl of RPMI 1640 supplemented with 10% FCS for 30 min. After the blocking solution was discarded, 100 µl of cell suspension was applied to the well (tear duct, 10^4 cells/well; spleen, 10^6 cells/well). After incubation for 4 h, the plates were washed three times with PBS, followed by three washes with 0.1% Tween-PBS. The plates were incubated with horseradish peroxidase–conjugated anti–mouse IgA or IgG (1:1,000 dilution [vol/vol] in 0.1% Tween-PBS) for 16 h. After the plate had been washed with PBS six times, antibody-producing cells were visualized with AECB-500 and AECM-100 conjugate solutions (Moss, Inc.). Plates were incubated for 30 min and washed with water. The plates were allowed to dry, and spot pictures were taken with a microscope. To detect CT-B–specific T cells, a CT-B/I-A^b tetramer was prepared and used for flow cytometric analysis, as previously described (Chang et al., 2008).

Cell preparation and RT-PCR. $CD3^-CD4^+CD45^+$ cells were isolated from mucosa-associated tissues as previously described (Fukuyama et al., 2006). In some experiments, mononuclear cells were isolated from the TALT, NALT, and NPs of immunized mice by mechanical dissociation (Fukuyama et al., 2002). Total RNA was extracted for RT-PCR as previously described (Shikina et al., 2004). The sequences of primers used were as follows: *Id2*, (sense) 5′-TCTGAGCTTATGTCGAATGATAGC-3′ and (anti-sense) 5′-CACAGCATTCAGTAGGCTCGTGTC-3′; *Rorγt*, (sense) 5′-ACCTCCACTGCCAGCTGTGTGCTGTC-3′ and (anti-sense) 5′-TTGTTTCTGCACTTCTGCATGTAGACTGTCCC-3′; *Gapdh*, (sense) 5′-TGAACGGGAAGCTCACTGG-3′ and (anti-sense) 5′-TCCACCACCCTGTTGCTGTA-3′; *Aid*, (sense) 5′-GGCTGAGGTTAGGGTTCCATCTCAG-3′ and (anti-sense) 5′-GAGGGAGTCAAGAAAGTCACGCTGGA-3′; and *β-actin*, (sense) 5′-TGGAATCCTGTGGCATCCATGAAA-3′ and (anti-sense) 5′-TAAAACGCAGCTCAGTAACAGTCC-3′.

Electron microscopy analysis. Electron microscopy was performed as previously described (Jang et al., 2004). Head tissue containing the tear ducts was prepared and fixed in a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde, and 0.1 M of sodium phosphate buffer (pH 7.6) on ice for 1 h. After washes with 4% sucrose in 0.1 M of phosphate buffer, the tissue was decalcified with 2.5% EDTA solution for 5 d. After three washes, the samples were fixed with 2% osmium tetraoxide on ice for 1 h and dehydrated with a series of ethanol gradients. For scanning electron microscopy, dehydrated tissues were freeze embedded in *t*-butyl alcohol and freeze dried, and then coated with osmium and observed under a scanning electron microscope (S-4200; Hitachi). For transmission electron microscopy, the tissues were embedded in Epon 812 resin mixture, and ultrathin (70-nm) sections were cut with an ultramicrotome (Reichert Ultracut N; Leica). The ultrathin sections were stained with 2% uranyl acetate in 70% ethanol for 5 min at room temperature and then in Reynold's lead for 5 min at room temperature. Sections were analyzed with a transmission electron microscope (H-7500; Hitachi).

Online supplemental material. Fig. S1 shows postnatal organogenesis of TALT on BALB/c mice. Fig. S2 shows development of TALT in B cell– or T cell–null mice and in TLR signaling–null conditions. Fig. S3 shows the lymphoid structure of TALT in mice lacking molecules related to lymphoid tissue genesis. Paraffinized sections were prepared from 8-wk-old mice and stained with the indicated antibodies (PNAd and B220) for confocal microscopy analysis. Arrows indicate PNAd-expressing HEVs. Fig. S4 shows the absence of MAdCAM-1 in TALT and NALT. TALT (A) and NALT (B) of 8-wk-old C57BL/6 mice were stained with DAPI and an anti–MAdCAM-1 antibody, and confocal microscopy analysis was performed. PPs of 10-d-old mice were analyzed as a positive control for anti–MAdCAM-1 antibody (C). Fig. S5 shows the independence of TALT genesis in TRANCE-Traf6 signaling. TALT of 2–3-wk-old *Trance*-deficient and *Traf6*, *Tcrb* double-deficient mice was analyzed histologically with hematoxylin and eosin (HE) staining. *Traf6*$^{+/-}$, *Tcrb*$^{-/-}$ mice were examined as a control. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091436/DC1.

We thank Dr. K. McGhee of the Medical College of Georgia for editorial help.

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), the Ministry of Health and Welfare of Japan and the Global Center of Excellence Program "Center of Education and Research for the Advanced Genome-Based Medicine For Personalized Medicine and the Control of Worldwide Infectious Diseases," and an "Academic Frontier" project for private universities matching-fund subsidy from MEXT. T. Nagatake, S. Fukuyama, and D.-Y. Kim were supported by research fellowships from the Japan Society for the Promotion of Science for Graduate Students, Young Scientists, and Foreign Researchers, respectively. A.M. Jetten was supported by the Intramural Research Program of the National Institute of Environmental Health Sciences (grant Z01-ES-101586).

The authors declare that they have no competing financial interests.

Submitted: 2 July 2009
Accepted: 16 September 2009

REFERENCES

Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998a. Targeted disruption of the *MyD88* gene results in loss of IL-1- and IL-18-mediated function. *Immunity*. 9:143–150. doi:10.1016/S1074-7613(00)80596-8

Adachi, S., H. Yoshida, K. Honda, K. Maki, K. Saijo, K. Ikuta, T. Saito, and S.-I. Nishikawa. 1998b. Essential role of IL-7 receptor α in the formation of Peyer's patch anlage. *Int. Immunol.* 10:1–6. doi:10.1093/intimm/10.1.1

Allansmith, M.R., J. Radl, J.J. Haaijman, and J. Mestecky. 1985. Molecular forms of tear IgA and distribution of IgA subclasses in human lacrimal glands. *J. Allergy Clin. Immunol.* 76:569–576. doi:10.1016/0091-6749(85)90777-8

Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Förster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature.* 406:309–314. doi:10.1038/35018581

Banks, T.A., B.T. Rouse, M.K. Kerley, P.J. Blair, V.L. Godfrey, N.A. Kuklin, D.M. Bouley, J. Thomas, S. Kanangat, and M.L. Mucenski. 1995. Lymphotoxin-α-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685–1693.

Boos, M.D., Y. Yokota, G. Eberl, and B.L. Kee. 2007. Mature natural killer cell and lymphoid tissue–inducing cell development requires Id2-mediated suppression of E protein activity. *J. Exp. Med.* 204:1119–1130. doi:10.1084/jem.20061959

Browning, J.L., N. Allaire, A. Ngam-Ek, E. Notidis, J. Hunt, S. Perrin, and R.A. Fava. 2005. Lymphotoxin-β receptor signaling is required for the homeostatic control of HEV differentiation and function. *Immunity.* 23:539–550. doi:10.1016/j.immuni.2005.10.002

Cain, C., and T.E. Phillips. 2008. Developmental changes in conjunctiva-associated lymphoid tissue of the rabbit. *Invest. Ophthalmol. Vis. Sci.* 49:644–649. doi:10.1167/iovs.07-0856

Chang, S.-Y., H.-R. Cha, S. Uematsu, S. Akira, O. Igarashi, H. Kiyono, and M.-N. Kweon. 2008. Colonic patches direct the cross-talk between systemic compartments and large intestine independently of innate immunity. *J. Immunol.* 180:1609–1618.

Chodosh, J., R.E. Nordquist, and R.C. Kennedy. 1998. Comparative anatomy of mammalian conjunctival lymphoid tissue: a putative mucosal immune site. *Dev. Comp. Immunol.* 22:621–630. doi:10.1016/S0145-305X(98)00022-6

Dejardin, E., N.M. Droin, M. Delhase, E. Haas, Y. Cao, C. Makris, Z.-W. Li, M. Karin, C.F. Ware, and D.R. Green. 2002. The lymphotoxin-β receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity.* 17:525–535. doi:10.1016/S1074-7613(02)00423-5

De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariathasan, S.C. Smith, R. Carlson, L.P. Shornick, J. Strauss-Schoenberger, et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science.* 264:703–707. doi:10.1126/science.8171322

Eberl, G., and D.R. Littman. 2004. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. *Science.* 305:248–251. doi:10.1126/science.1096472

Eberl, G., S. Marmon, M.J. Sunshine, P.D. Rennert, Y. Choi, and D.R. Littman. 2004. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat. Immunol.* 5:64–73. doi:10.1038/ni1022

Ebisuno, Y., T. Tanaka, N. Kanemitsu, H. Kanda, K. Yamaguchi, T. Kaisho, S. Akira, and M. Miyasaka. 2003. Cutting edge: the B cell chemokine CXC chemokine ligand 13/B lymphocyte chemoattractant is expressed in the high endothelial venules of lymph nodes and Peyer's patches and affects B cell trafficking across high endothelial venules. *J. Immunol.* 171:1642–1646.

Fukuyama, S., T. Hiroi, Y. Yokota, P.D. Rennert, M. Yanagita, N. Kinoshita, S. Terawaki, T. Shikina, M. Yamamoto, Y. Kurono, and H. Kiyono. 2002. Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(−)CD4(+)CD45(+) cells. *Immunity.* 17:31–40. doi:10.1016/S1074-7613(02)00339-4

Fukuyama, S., T. Nagatake, D.-Y. Kim, K. Takamura, E.J. Park, T. Kaisho, N. Tanaka, Y. Kurono, and H. Kiyono. 2006. Cutting edge: Uniqueness of lymphoid chemokine requirement for the initiation and maturation of nasopharynx-associated lymphoid tissue organogenesis. *J. Immunol.* 177:4276–4280.

Giuliano, E.A., C.P. Moore, and T.E. Phillips. 2002. Morphological evidence of M cells in healthy canine conjunctiva-associated lymphoid tissue. *Graefes Arch. Clin. Exp. Ophthalmol.* 240:220–226. doi:10.1007/s00417-002-0429-3

Gomes, J.A.P., V.K. Jindal, P.D. Gormley, and H.S. Dua. 1997. Phenotypic analysis of resident lymphoid cells in the conjunctiva and adnexal tissues of rat. *Exp. Eye Res.* 64:991–997. doi:10.1006/exer.1997.0297

Gupta, A., D. Monroy, Z. Ji, K. Yoshino, A. Huang, and S.C. Pflugfelder. 1996. Transforming growth factor beta-1 and beta-2 in human tear fluid. *Curr. Eye Res.* 15:605–614. doi:10.3109/02713689609008900

Hamada, H., T. Hiroi, Y. Nishiyama, H. Takahashi, Y. Masunaga, S. Hachimura, S. Kaminogawa, H. Takahashi-Iwanaga, T. Iwanaga, H. Kiyono, et al. 2002. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol.* 168:57–64.

Harmsen, A., K. Kusser, L. Hartson, M. Tighe, M.J. Sunshine, J.D. Sedgwick, Y. Choi, D.R. Littman, and T.D. Randall. 2002. Cutting edge: organogenesis of nasal-associated lymphoid tissue (NALT) occurs independently of lymphotoxin-α (LT α) and retinoic acid receptor-related orphan receptor-γ, but the organization of NALT is LT α dependent. *J. Immunol.* 168:986–990.

Haynes, R.J., P.J. Tighe, and H.S. Dua. 1998. Innate defence of the eye by antimicrobial defensin peptides. *Lancet.* 352:451–452. doi:10.1016/S0140-6736(05)79185-6

Hazlett, L.D., S. McClellan, R. Barrett, and X. Rudner. 2001. B7/CD28 costimulation is critical in susceptibility to *Pseudomonas aeruginosa* corneal infection: a comparative study using monoclonal antibody blockade and CD28-deficient mice. *J. Immunol.* 166:1292–1299.

Honda, K., H. Nakano, H. Yoshida, S. Nishikawa, P. Rennert, K. Ikuta, M. Tamechika, K. Yamaguchi, T. Fukumoto, T. Chiba, and S.-I. Nishikawa. 2001. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *J. Exp. Med.* 193:621–630. doi:10.1084/jem.193.5.621

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol.* 162:3749–3752.

Jang, M.H., M.N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P.D. Rennert, et al. 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc. Natl. Acad. Sci. USA.* 101:6110–6115. doi:10.1073/pnas.0400969101

Kanamori, Y., K. Ishimaru, M. Nanno, K. Maki, K. Ikuta, H. Nariuchi, and H. Ishikawa. 1996. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit$^+$ IL-7R$^+$ Thy1$^+$ lympho-hemopoietic progenitors develop. *J. Exp. Med.* 184:1449–1459. doi:10.1084/jem.184.4.1449

Kelsoe, G. 1996. Life and death in germinal centers (redux). *Immunity.* 4:107–111. doi:10.1016/S1074-7613(00)80675-5

Kijlstra, A. 1990. The role of lactoferrin in the nonspecific immune response on the ocular surface. *Reg. Immunol.* 3:193–197.

Kiyono, H., and S. Fukuyama. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4:699–710. doi:10.1038/nri1439

Knop, N., and E. Knop. 2000. Conjunctiva-associated lymphoid tissue in the human eye. *Invest. Ophthalmol. Vis. Sci.* 41:1270–1279.

Knop, E., and N. Knop. 2001. Lacrimal drainage-associated lymphoid tissue (LDALT): a part of the human mucosal immune system. *Invest. Ophthalmol. Vis. Sci.* 42:566–574.

Knop, N., and E. Knop. 2005. Ultrastructural anatomy of CALT follicles in the rabbit reveals characteristics of M-cells, germinal centres and high endothelial venules. *J. Anat.* 207:409–426. doi:10.1111/j.1469-7580.2005.00470.x

Kong, Y.Y., H. Yoshida, I. Sarosi, H.-L. Tan, E. Timms, C. Capparelli, S. Morony, A.J. Oliveira-dos-Santos, G. Van, A. Itie, et al. 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature.* 397:315–323. doi:10.1038/16852

Kurebayashi, S., E. Ueda, M. Sakaue, D.D. Patel, A. Medvedev, F. Zhang, and A.M. Jetten. 2000. Retinoid-related orphan receptor γ

(RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proc. Natl. Acad. Sci. USA.* 97:10132–10137. doi:10.1073/pnas.97.18.10132

Liesegang, T.J. 1998. Bacterial and fungal keratitis. *In* The Cornea. H.E. Kaufman, B.A. Barron, M.B. McDonald, and S.R. Waltman, editors. Churchill Livingstone, New York. 217–270.

Luther, S.A., K.M. Ansel, and J.G. Cyster. 2003. Overlapping roles of CXCL13, interleukin 7 receptor α, and CCR7 ligands in lymph node development. *J. Exp. Med.* 197:1191–1198. doi:10.1084/jem.20021294

Mebius, R.E. 2003. Organogenesis of lymphoid tissues. *Nat. Rev. Immunol.* 3:292–303. doi:10.1038/nri1054

Mebius, R.E., P. Rennert, and I.L. Weissman. 1997. Developing lymph nodes collect CD4+CD3− LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity.* 7:493–504. doi:10.1016/S1074-7613(00)80371-4

Mestecky, J., R. Blumberg, H. Kiyono, and J.R. McGhee. 2003. Chapter 31. *In* Fundamental Immunology. Fifth edition. W.E. Paul, editor. Academic Press, San Diego. 965–1020.

Naito, A., S. Azuma, S. Tanaka, T. Miyazaki, S. Takaki, K. Takatsu, K. Nakao, K. Nakamura, M. Katsuki, T. Yamamoto, and J. Inoue. 1999. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in *TRAF6*-deficient mice. *Genes Cells.* 4:353–362. doi:10.1046/j.1365-2443.1999.00265.x

Naito, T., T. Shiohara, T. Hibi, M. Suematsu, and H. Ishikawa. 2008. ROR γ t is dispensable for the development of intestinal mucosal T cells. *Mucosal Immunol.* 1:198–207. doi:10.1038/mi.2008.4

Nakamura, Y., C. Sotozono, and S. Kinoshita. 1998. Inflammatory cytokines in normal human tears. *Curr. Eye Res.* 17:673–676. doi:10.1080/02713689808951242

Nakano, H., T. Tamura, T. Yoshimoto, H. Yagita, M. Miyasaka, E.C. Butcher, H. Nariuchi, T. Kakiuchi, and A. Matsuzawa. 1997. Genetic defect in T lymphocyte-specific homing into peripheral lymph nodes. *Eur. J. Immunol.* 27:215–221. doi:10.1002/eji.1830270132

Nakano, H., S. Mori, H. Yonekawa, H. Nariuchi, A. Matsuzawa, and T. Kakiuchi. 1998. A novel mutant gene involved in T-lymphocyte-specific homing into peripheral lymphoid organs on mouse chromosome 4. *Blood.* 91:2886–2895.

Nochi, T., Y. Yuki, A. Matsumura, M. Mejima, K. Terahara, D.-Y. Kim, S. Fukuyama, K. Iwatsuki-Horimoto, Y. Kawaoka, T. Kohda, et al. 2007. A novel M cell–specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses. *J. Exp. Med.* 204:2789–2796. doi:10.1084/jem.20070607

Parks, Q.M., and J.A. Hobden. 2005. Polyphosphate kinase 1 and the ocular virulence of *Pseudomonas aeruginosa*. *Invest. Ophthalmol. Vis. Sci.* 46:248–251. doi:10.1167/iovs.04-0340

Paulsen, F.P., J.I. Paulsen, A.B. Thale, and B.N. Tillmann. 2000. Mucosa-associated lymphoid tissue in human efferent tear ducts. *Virchows Arch.* 437:185–189. doi:10.1007/s004280000248

Paulsen, F.P., U. Schaudig, S. Maune, and A.B. Thale. 2003. Loss of tear duct-associated lymphoid tissue in association with the scarring of symptomatic dacryostenosis. *Ophthalmology.* 110:85–92. doi:10.1016/S0161-6420(02)01442-2

Peppard, J.V., and P.C. Montgomery. 1987. Studies on the origin and composition of IgA in rat tears. *Immunology.* 62:193–198.

Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor–deficient mice. *J. Exp. Med.* 180:1955–1960. doi:10.1084/jem.180.5.1955

Rangel-Moreno, J., J. Moyron-Quiroz, K. Kusser, L. Hartson, H. Nakano, and T.D. Randall. 2005. Role of CXC chemokine ligand 13, CC chemokine ligand (CCL) 19, and CCL21 in the organization and function of nasal-associated lymphoid tissue. *J. Immunol.* 175:4904–4913.

Rangel-Moreno, J., J.E. Moyron-Quiroz, D.M. Carragher, K. Kusser, L. Hartson, A. Moquin, and T.D. Randall. 2009. Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell response to peritoneal antigens. *Immunity.* 30:731–743. doi:10.1016/j.immuni.2009.03.014

Rennert, P.D., D. James, F. Mackay, J.L. Browning, and P.S. Hochman. 1998. Lymph node genesis is induced by signaling through the lymphotoxin β receptor. *Immunity.* 9:71–79. doi:10.1016/S1074-7613(00)80589-0

Ridley Lathers, D.M., R.F. Gill, and P.C. Montgomery. 1998. Inductive pathways leading to rat tear IgA antibody responses. *Invest. Ophthalmol. Vis. Sci.* 39:1005–1011.

Saitoh-Inagawa, W., T. Hiroi, M. Yanagita, H. Iijima, E. Uchio, S. Ohno, K. Aoki, and H. Kiyono. 2000. Unique characteristics of lacrimal glands as a part of mucosal immune network: high frequency of IgA-committed B-1 cells and NK1.1+ alphabeta T cells. *Invest. Ophthalmol. Vis. Sci.* 41:138–144.

Shapiro-Shelef, M., and K. Calame. 2005. Regulation of plasma-cell development. *Nat. Rev. Immunol.* 5:230–242. doi:10.1038/nri1572

Shikina, T., T. Hiroi, K. Iwatani, M.H. Jang, S. Fukuyama, M. Tamura, T. Kubo, H. Ishikawa, and H. Kiyono. 2004. IgA class switch occurs in the organized nasopharynx- and gut-associated lymphoid tissue, but not in the diffuse lamina propria of airways and gut. *J. Immunol.* 172:6259–6264.

Shinkura, R., K. Kitada, F. Matsuda, K. Tashiro, K. Ikuta, M. Suzuki, K. Kogishi, T. Serikawa, and T. Honjo. 1999. Alymphoplasia is caused by a point mutation in the mouse gene encoding Nf-κ b-inducing kinase. *Nat. Genet.* 22:74–77. doi:10.1038/8780

Stein-Streilein, J., and A.W. Taylor. 2007. An eye's view of T regulatory cells. *J. Leukoc. Biol.* 81:593–598. doi:10.1189/jlb.0606383

Sullivan, D.A., and M.R. Allansmith. 1984. Source of IgA in tears of rats. *Immunology.* 53:791–799.

Sun, Z., D. Unutmaz, Y.R. Zou, M.J. Sunshine, A. Pierani, S. Brenner-Morton, R.E. Mebius, and D.R. Littman. 2000. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science.* 288:2369–2373. doi:10.1126/science.288.5475.2369

Suzuki, K., T. Oida, H. Hamada, O. Hitotsumatsu, M. Watanabe, T. Hibi, H. Yamamoto, E. Kubota, S. Kaminogawa, and H. Ishikawa. 2000. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. *Immunity.* 13:691–702. doi:10.1016/S1074-7613(00)00068-6

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11:443–451. doi:10.1016/S1074-7613(00)80119-3

Taylor, R.T., A. Lügering, K.A. Newell, and I.R. Williams. 2004. Intestinal cryptopatch formation in mice requires lymphotoxin α and the lymphotoxin β receptor. *J. Immunol.* 173:7183–7189.

Tsuji, M., K. Suzuki, H. Kitamura, M. Maruya, K. Kinoshita, I.I. Ivanov, K. Itoh, D.R. Littman, and S. Fagarasan. 2008. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. *Immunity.* 29:261–271. doi:10.1016/j.immuni.2008.05.014

Uchio, E., S.Y. Ono, Z. Ikezawa, and S. Ohno. 2000. Tear levels of interferon-gamma, interleukin (IL)-2, IL-4 and IL-5 in patients with vernal keratoconjunctivitis, atopic keratoconjunctivitis and allergic conjunctivitis. *Clin. Exp. Allergy.* 30:103–109. doi:10.1046/j.1365-2222.2000.00699.x

Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature.* 397:702–706. doi:10.1038/17812

Original Paper

International Archives of
Allergy and Immunology

Int Arch Allergy Immunol 036
DOI: 10.1159/000XXXXXX

Received: August 18, 2008
Accepted after revision: January 15, 2009

Suppression of Allergic Diarrhea in Murine Ovalbumin-Induced Allergic Diarrhea Model by PG102, a Water-Soluble Extract Prepared from *Actinidia arguta*

Donghyun Kim[a] Seon Hee Kim[b] Eun-Jin Park[b] Jiyoung Kim[a]
Sang-Heon Cho[c] Junko Kagawa[d] Naoko Arai[d] Kunisawa Jun[e]
Hiroshi Kiyono[e] Sunyoung Kim[a]

[a]School of Biological Sciences, Seoul National University, [b]Helixir Co. Ltd., Biotechnology Incubating Center, Seoul National University and [c]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea; [d]SBI Biotech Co. Ltd., Ginkgo Biomedical Research Institute, Kawasaki, and [e]Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Key Words
Actinidia arguta · Antiallergy · Diarrhea · Food allergy · IgE · IL-6 · IL-10 · MCP-1 · PG102

Abstract

Background: Allergic reactions to food can involve diarrhea, vomiting, nausea and abnormal pain. PG102 has previously been shown to control various factors involved in allergy pathogenesis, including IgE and various Th1 and Th2 cytokines, in vivo as well as in vitro [Park EJ, et al.: J Allergy Clin Immunol 2005;116:1151–1157; Park EJ, et al.: J Invest Dermatol 2007;127:1154–1160]. These data indicate that PG102 might have antiallergic effects on allergic diarrhea. Here, we investigated whether PG102 could prevent allergic diarrhea in the murine ovalbumin (OVA)-induced allergic diarrhea model. ***Methods:*** BALB/c mice were orally treated with PG102, dexamethasone or water for 9 days on a daily basis, followed by subcutaneous injection with OVA on day 0. Animals were orally administrated with OVA from day 7, 3 times a week, over a period of approximately 20 days. Incidence of diarrhea, serum, OVA-restimulated splenocytes and lamina propria lymphocytes were analyzed. ***Results:*** Oral administration of PG102 could suppress the incidence of diarrhea in a murine allergic diarrhea model. The amelioration of allergic diarrhea by PG102 was accompanied with the inhibition of mast cell infiltration into the large intestine. The serum level of IgE, IL-6 and MCP-1 was decreased in PG102-treated mice. When splenocytes were isolated from respective groups and cultured in the presence of OVA, cells from PG102-administrated animals produced lesser amounts of IL-6 and MCP-1. ***Conclusions:*** PG102 has the potential to be used as a preventive for food allergic diseases.

Introduction

Diarrhea is a representative intestinal allergic symptom resulting from the abnormal absorption of nutrients and water and/or the intestinal secretion of fluid by inflammatory responses [1]. Most common food allergies are caused by IgE-mediated reactions to food, known as type I hypersensitivity reactions [2–4]. In the susceptible individual, allergens processed by antigen-presenting cells promote the differentiation of naïve T helper (Th0)

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–2438/09/0000–0000$26.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Sunyoung Kim
School of Biological Sciences, Laboratory of Virology
Building 504, Seoul National University
Gwanak-gu, Seoul 151-742 (Korea)
Tel. +82 2 880 8015, Fax +82 2 875 0907, E-Mail sunyoung@snu.ac.kr

cells to Th2 phenotype and lead to the induction of an isotype switching to IgE production in plasma cells [3, 4]. IgE can tightly bind to high-affinity receptors, FcεRI, present on mast cells and basophils in the tissue and the blood. Binding of the antigen to IgE cross-links these receptors, which provokes activation of the mast cells and the basophils, resulting in the releases of preformed and newly synthesized inflammatory molecules, such as histamine, leukotrienes, cytokines and chemokines [1, 3]. Thereafter, the released mediators recruit more leukocytes including eosinophils, mast cells, basophils and Th2 lymphocytes to the site of the allergic inflammation. These cells are major contributors to a chronic allergic inflammation, triggering tissue damage [3, 4].

We previously found that PG102, a water-soluble extract from *Actinidia arguta*, commonly called hardy kiwifruit, modulates the level of IgE and Th1/Th2 cytokines in in vitro cell culture systems as well as in 3 different allergy models [5, 6, submitted]. It was subsequently found that oral administration of PG102 could improve asthma conditions in a murine model, probably by regulating the level of IgE and IL-5 [7]. In these experiments, PG102 was orally administrated after [5] or simultaneously with [6, 7] induction of the allergy. All these in vitro and in vivo data strongly suggested that PG102 might be an effective antiallergic agent for various allergic diseases. In this study, a murine OVA-induced diarrhea model was used for testing the preventive effects of PG102 on food allergies. Our data indicated that oral administration reproducibly and effectively suppressed the incidence of diarrhea. PG102 regulated various molecules related to allergic reactions, IgE, IL-6 and MCP-1 in the serum, and IL-6, IL-10 and MCP-1 in ovalbumin (OVA)-restimulated splenocytes. Furthermore, the recruitment of mast cells into the large intestine was found to be inhibited in PG102-treated animals. These results suggested that oral administration of PG102 might be a useful preventive and/or therapeutic for food allergies as well as other allergic diseases such as dermatitis and asthma.

Material and Methods

Preparation of PG102

The hardy kiwifruits used in this study were purchased from a company specializing in this fruit (Vital Berry Marketing SA, Santiago, Chile). Total water-soluble extract of the dried fruits, PG102T in our previous reports [5], has been named PG102 in this study. PG102 was prepared from these hardy kiwifruits as described previously [5]. Briefly, the dried fruits were extracted by boiling in distilled water for 3 h. The extract was filtered with Whatman filter paper (No. 2, 110 nm), and concentrated using a rotary evaporator, followed by a freeze-drying process. Powdered PG102 was dissolved in distilled water at a concentration of 200 mg/ml and stored at –80°C until use. The dry form of PG102 contains 2.4% protein (as determined by semimicro-Kjeldahl method), 91.6% carbohydrate (by phenol-surfuric acid method), 182 IU/100 g vitamin A (by SbCl3 method) and 680 mg/100 g vitamin C (by DNP method). Lipid was not detected, and acidity of PG102 was pH 7.4 as lactic acid. All PG102 preparations contained undetectable levels of Gram-negative bacterial endotoxin, as determined by the limulus amebocytes lysate assay (Cambrex, [redacted], Md., USA). PG102 was also tested to be negative for heavy metals, residual pesticides and microorganisms.

Bioassay for PG102

To determinate the biological activity of PG102, RBL-2H3 cells, a rat mast cell line, were plated at 2 × 10^5 cells/well in a 24-well culture plate and grown in 0.5 ml MEM (Sigma, St. Louis, Mo., USA) supplemented with 15% FBS (Gibco, Grand Island, N.Y., USA) at 37°C under 5% CO_2 for 6 h. Cells were treated with various concentrations of PG102 for 30 min before stimulation with 1 mM of A23187 (Sigma). Twelve hours later, the supernatants were taken to measure the level of IL-4 by ELISA (R&D Systems, Minneapolis, Minn., USA).

Murine Allergic Diarrhea Model

Female BALB/c mice (6 weeks old) were purchased from Orientbio Inc. (Seongnam, Korea), bred under aseptic conditions in facilities at Seoul National University, and acclimated for at least 1 week before use. All experimental procedures were performed in compliance with the guidelines set by the University Animal Care and Use Committee at Seoul National University. Two groups of mice were gastrically administered with PG102 (200 mg/kg/day) or dexamethasone (Dex; 2.5 mg/kg/day; Sigma) in the volume of 200 μl, from day –9 to day 26 (or to day 33, depending on the status of diarrhea induction), while another group of mice was fed with 200 μl of water alone. As another control, normal mice were fed with 200 μl of water without immunization. The first 3 groups of mice were sensitized by subcutaneous injection with 1 mg OVA (Fraction V; Sigma) emulsified in 100 μl complete Freund's adjuvant (Sigma) on day 0. One week after sensitization, mice were orally administered with 100 mg of OVA dissolved in 300 μl of PBS, 3 times per week for 3 or 4 weeks, depending on the experiments. Two hours following the last administration, the mice were sacrificed and sera, spleens as well as intestines were obtained [8, 9].

Isolation and Culturing of Splenocytes

Splenocytes were prepared by the mechanical dissociation method [9] and resuspended in RPMI-1640 (Sigma) containing 10% FBS (Gibco). Splenocytes were seeded at 5 × 10^6 cells/ml/well, and incubated with 100 μg/ml of OVA for 3 days [5, 6, 9]. Collected culture supernatants were used for the analysis of inflammatory mediators.

Measurement of Immunoglobulins, Cytokines and Chemokines

The total level of IgE was determined using a mouse IgE detection kit (Shibayagi, Gunma, Japan). The level of OVA-specific IgE was measured by the sandwich ELISA method (BD Biosciences, San Jose, Calif., USA; Pierce, Rockford, Ill., USA) as described by

Hirano et al. [5, 6, 10]. The levels of IL-6, IL-10 and MCP-1 in the serum and the restimulated splenocytes were measured by commercially available ELISA kits (Pierce; R&D Systems).

Measurement of Mast Cell Infiltration into Large Intestine

Mononuclear cells were dissociated from the large intestines using 0.5 mg/ml of collagenase (type IV; Sigma) and purified using a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) as described by Kweon et al. [8, 9]. The separated cells were pooled and labeled by the PE-conjugated anti-CD117 antibody and the FITC-conjugated mouse IgE (BD Biosciences). The labeled cells were analyzed by flow cytometry analysis using a FACS caliber (BD Biosciences) [5, 8, 11].

Statistics

Data are presented as the means ± SEM and evaluated by the Student's t test for unpaired samples. $p < 0.05$ was considered to be statistically significant.

Results

Preparation of PG102 from A. arguta

A water-soluble extract, code-named PG102, was prepared from *A. arguta*, as described previously [5]. PG102 has previously been shown to regulate the expression of Th1 and Th2 cytokines as well as that of IgE [5, 6]. The active compounds contributing to these properties have not yet been precisely identified. To quantitatively perform experiments and also to obtain an experimental reagent in a consistent manner, reliable bioassay systems have been developed. One such assay is the use of a cell line together with IL-4 as a biomarker for the quality control of PG102. IL-4 plays key roles in the pathogenesis of almost all allergies [4]. The rat mast cell line, RBL-2H3, was treated with various concentrations of PG102, in the presence of calcium ionophore, A23187. When cells were treated with A23187, the level of IL-4 was highly increased from the undetectable basal level. The production of IL-4 was inhibited by PG102 in a dose-dependent manner (fig. 1). In all concentrations used in this assay, no cytotoxic effects were found. The IC_{50} value of PG102 used in this study was 1.50 mg/ml. This method was used for the quality control of experimental samples as well as the study of the molecular mechanism underlying the effects of PG102.

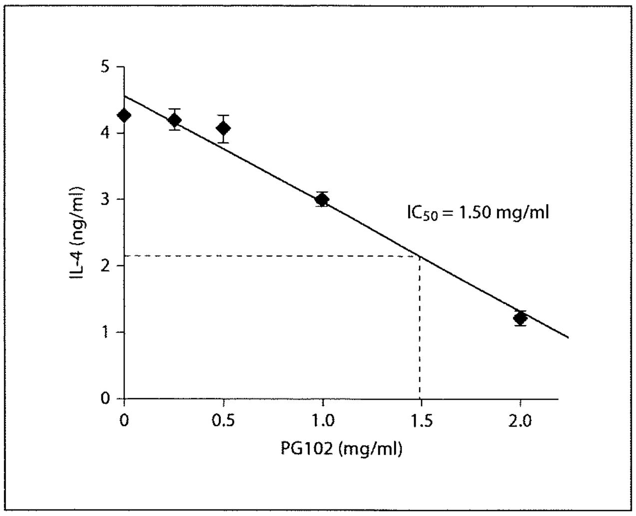


Fig. 1. Effects of PG102 in the bioassay system using RBL-2H3 cells. Rat RBL-2H3 mast cells were stimulated with PG102 (0, 0.5, 1 and 2 mg/ml) and A23187 (1 μM). Twelve hours later, the level of IL-4 was measured in the supernatant by ELISA kit. The results are shown as means ± SD measured in the duplicate microplate wells. More than 3 independent sets of experiments were performed.

Oral Administration of PG102 Suppressed Antigen-Specific Allergic Diarrhea

It was first examined whether oral administration of PG102 could prevent food allergy conditions using murine OVA-induced allergic diarrhea model [8]. BALB/c mice were sensitized once with OVA by subcutaneous injection on day 0, and intragastrically administrated with excess OVA 3 times a week from day 7 to the sacrifice day. For the purpose of comparison, animals were divided into 3 groups, each orally treated with PG102, Dex or water alone, once a day from day –9 to the sacrifice day, as shown in figure 2. As another control, 1 group of mice was grown without OVA immunization and with water administration only.

When animals were orally administrated with OVA from day 7, water-treated and OVA-immunized mice (OVA/water group) began to experience loosening of the bowels from the second treatment. After the eighth oral challenge on day 24, all mice from the OVA/water group suffered from severe diarrhea (fig. 3). In the PG102-treated and immunized group (OVA/PG102 group), the first lax stools were observed at the sixth oral administration with OVA, and at the end of the experiment, the symptom of diarrhea was observed in only 20–40% of mice in 4 independent experiments (fig. 3). No diarrhea-related symptom was observed in the nonsensitized animals (normal group) and the Dex-treated and immunized mice (OVA/Dex group; fig. 3). Although Dex completely blocked the induction of allergic diarrhea, mice treated with this glucocorticoid showed significant side effects, including reduction of body weight by more than 10%

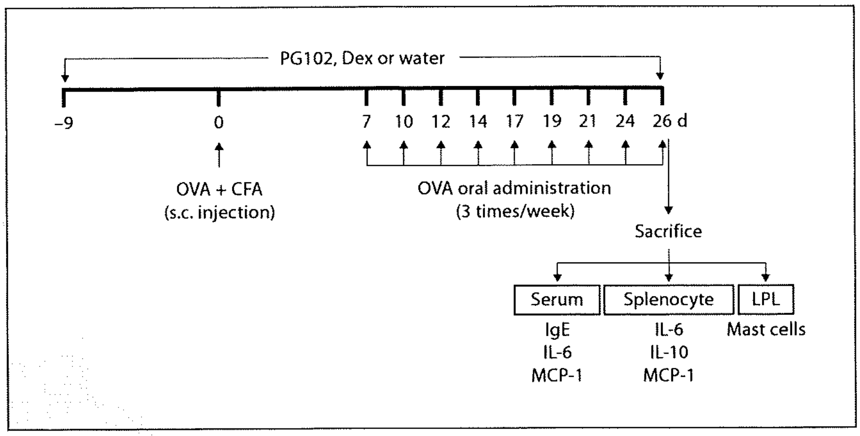
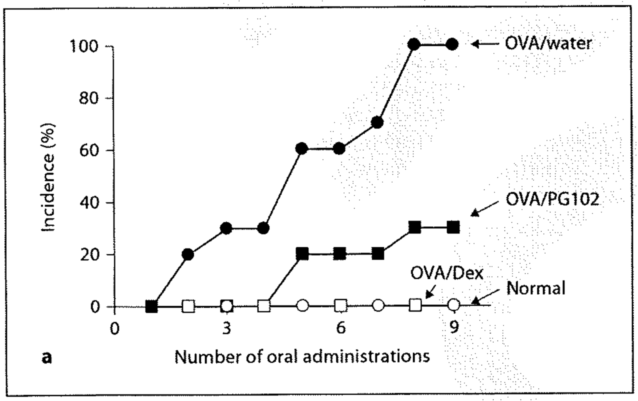
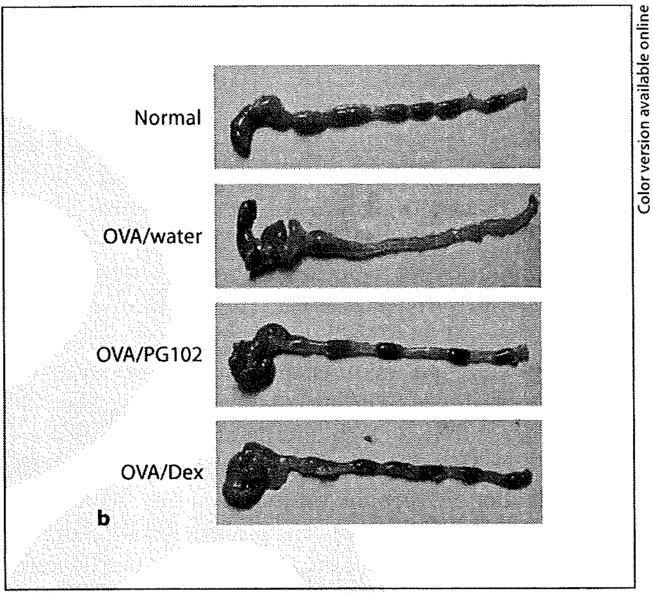


Fig. 2. Protocols for OVA sensitization, challenge and PG102 administration. Mice were sensitized by subcutaneous (s.c.) injection with 1 mg of OVA plus CFA on day 0, and then orally challenged with 100 mg of OVA 3 times a week. Animals were divided into 3 groups, and administered with PG102, Dex or water, on a daily basis from day –9 to day 26. As a control, 1 group of mice was fed with water only without OVA sensitization. Between 30 min and 2 h after each oral challenge with OVA, mice were observed to determine whether they had loose stools or not. On day 26, mice were sacrificed and samples were analyzed.





Color version available online

Fig. 3. Effects of PG102 in allergic diarrhea. Diarrhea was induced by OVA sensitization and repeated challenges (10 mice/group). **a** The effect on diarrhea incidence is shown. **b** The effect on macroscopic status of large intestine is shown. This figure provides 1 representative result of 4 separate sets of experiments.

and serious loss of thymus (data not shown). On the contrary, mice administered with PG102 maintained regular body weights, as compared with that of OVA/water as well as normal groups, and did not show any noticeable side effects (data not shown). These data indicated that oral administration of PG102 could ameliorate allergic diarrhea without side effects.

Effect on the Serum Level of IgE, IL-6 and MCP-1

Allergen-specific IgE mediates a type I hypersensitivity, triggering inflammatory responses in the local tissue and eventually generating various allergic symptoms, including food allergy [2–4, 12]. Next, we examined the serum level of total and OVA-specific IgE, IL-6 and MCP-1, which are all known to play key roles in the establishment of allergic diseases and related inflammatory reactions [2, 4, 13–15]. The immunization with OVA increased the level of total and OVA-specific IgE in a statistically significant manner, compared with that from normal mice (fig. 4a and b). More than 30% reduction in the level of total IgE was observed in OVA/PG102 animals. In particular, it is worth noting that the magnitude of OVA-specific IgE suppression mediated by PG102 appeared to be similar to that in the OVA/Dex group (fig. 4b). The serum level of IL-6 and MCP-1 was also dramatically increased by 3- to 4-fold when animals were immunized with OVA.

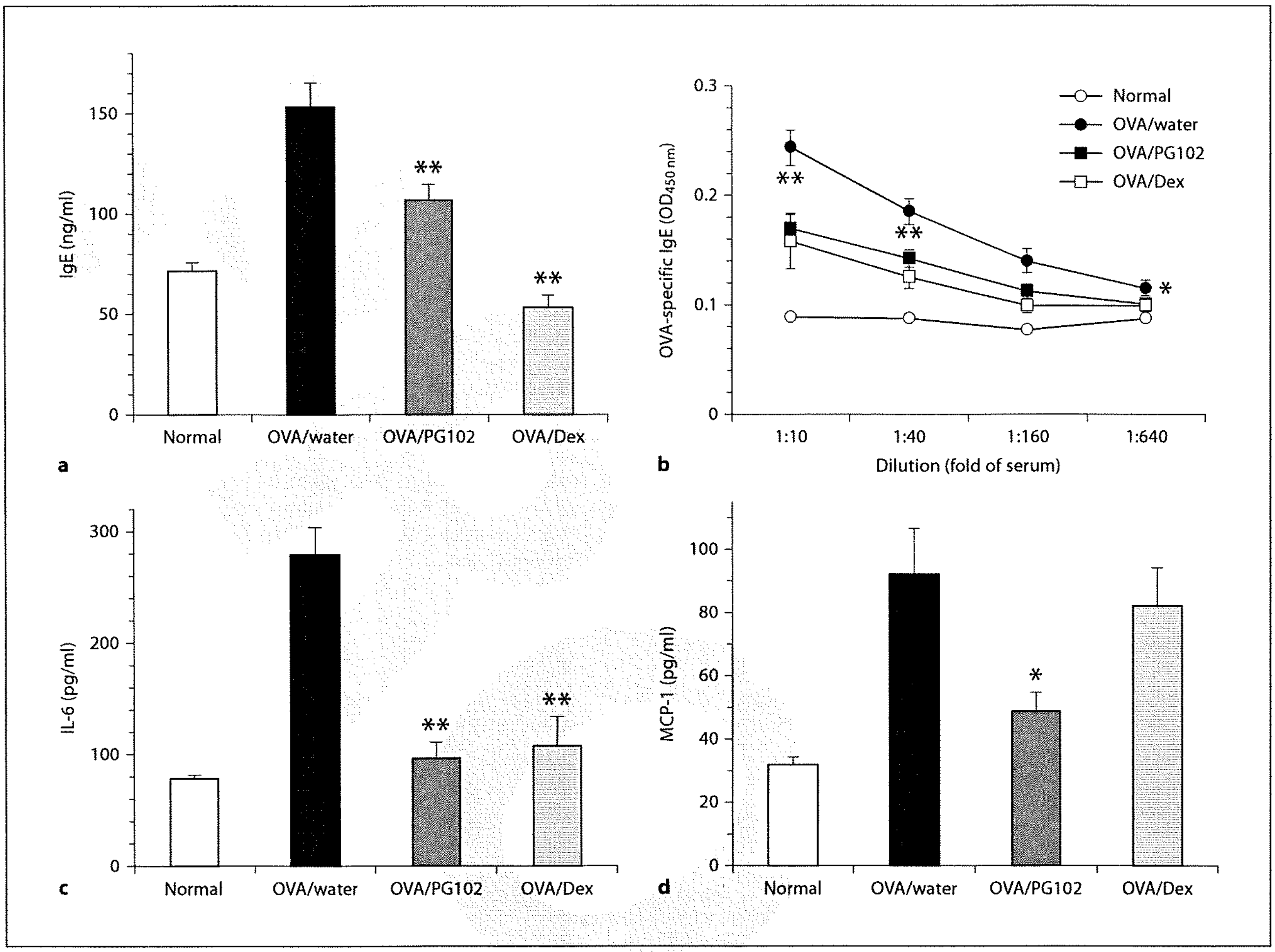


Fig. 4. Effects of PG102 on the serum level of total and OVA-specific IgE, IL-6 and MCP-1. **a**, **c**, **d** The level of total IgE, IL-6 and MCP-1 in the serum was determined using respective ELISA kits. **b** Because OVA-specific IgE antibody is unavailable, the relative level of OVA-specific IgE was represented as absorbance at $OD_{450\ nm}$ using a manual ELISA method. Values are shown as means ± SEM. * $p < 0.05$, ** $p < 0.01$ vs. OVA/water mice (Student's t test).

The production of IL-6 was lowered by approximately 3-fold in both OVA/PG102 and OVA/Dex groups, returning to an almost normal level (fig. 4c). In the case of MCP-1, only PG102 but not Dex could reduce its level (fig. 4d). These data suggested that PG102 could downregulate the serum level of 3 key factors involved in the early and late stages of allergic disease.

Effects of PG102 on Cytokine and Chemokine Production in Splenocytes from OVA-Sensitized Mice

To be certain that the above observation could be reproduced in an in vitro system, splenocytes were isolated from the mice of each group and cultured with OVA for 3 days. The culture supernatants were taken to measure the level of IL-6, IL-10 and MCP-1. Splenocytes isolated from the OVA/water group produced a large amount of IL-6, IL-10 and MCP-1 (fig. 5). However, cells from PG102-treated mice showed a significantly lower level of IL-6 (fig. 5a). The level of MCP-1 was also decreased, though the difference was not statistically significant (fig. 5b). The suppressive effect of PG102 on these inflammatory mediators was comparable to that of Dex (fig. 5a and b). On the contrary, the level of IL-10 produced from the splenocytes of PG102-fed mice was higher than that of the OVA/water group, while it was lower in the OVA/Dex group (fig. 5c). These data suggested that PG102

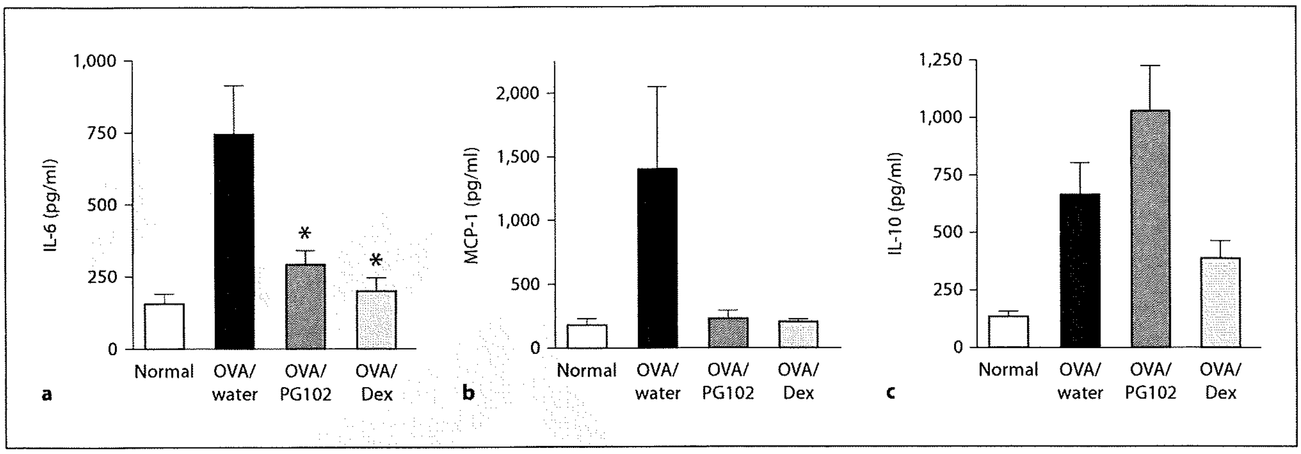
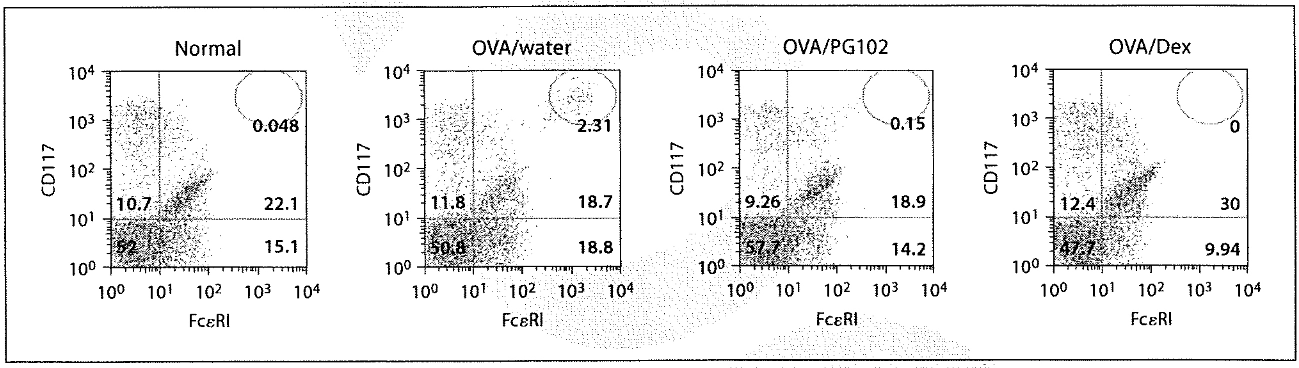


Fig. 5. Effect of PG102 on the production of IL-6, IL-10 and MCP-1 in OVA-restimulated splenocytes. Isolated splenocytes were cultured in the presence of 100 μg/ml of OVA for 3 days. The culture supernatants were taken to measure the level of IL-6 (**a**), MCP-1 (**b**) and IL-10 (**c**) using respective ELISA kits. The results are presented as means ± SEM. * $p < 0.05$ vs. OVA/water mice (Student's t test).



Color version available online

Fig. 6. Analysis of mast cell (CD117^{hi}FcεRI^{hi}) infiltration into large intestine. LPLs were isolated from the large intestines, labeled with dye-conjugated antibodies specific to CD117 and FcεRI, and analyzed by flow cytometry. The upper right corner of CD117^{hi}FcεRI^{hi} represents mast cells that infiltrated into the large intestine. This result is representative of 2 independent experiments each containing 2 mice.

might control pro- and anti-inflammatory molecules in a selective manner, unlike Dex, which suppressed the production of cytokines in an indiscriminate manner.

Oral Administration of PG102 Inhibits the Infiltration of CD117^{hi}FcεRI^{hi} Mast Cells into the Large Intestine

The lamina propria is a constituent layer of mucosa. Many different kinds of leukocytes exist in this region, and they play important roles in inducing various immune reactions to food [12, 16]. To test the infiltration status of mast cells in the large intestine, lamina propria lymphocytes (LPLs) were isolated and analyzed by flow cytometry [8, 9, 11]. Mast cells highly express CD117 and FcεRI on their surface [11]. As revealed in figure 6, the proportion of infiltrating mast cells (CD117^{hi}FcεRI^{hi}) in the large intestine was increased to 2.31% in OVA/water mice from 0.048% in normal mice. However, oral administration of PG102 and Dex decreased the fraction of mast cells to 0.15 and 0%, respectively (fig. 6). These results indicated that PG102 might suppress an allergic inflammation by inhibiting the infiltration of CD117^{hi}FcεRI^{hi} mast cells into the allergen-sensitized large intestine.

Discussion

Specific foods can provoke adverse reactions in a susceptible individual by hypersensitivity reaction, which is termed food allergy [1–3]. Food allergens are derived from a limited number of foods, such as cow's milk, eggs, soy, wheat, peanuts and seafood, so the management of food allergy generally focuses on the avoidance of such causal food(s) from the diet [2, 3, 12]. However, the elimination of ubiquitous allergens from food is very difficult and time-consuming, and even careful individuals may accidentally ingest causal food(s) through cross-contamination. A recent clinical trial showed that the use of humanized monoclonal anti-IgE antibody, called TNX-901, could significantly increase the threshold for individuals' sensitivity to peanuts [17]. Theoretically, this approach should be protective against multiple food allergens, however, it requires continued treatment and is too expensive to be used for the purpose of prevention. Therefore, there has been a strong need for the development of a convenient, cost-effective and efficacious preventive or therapeutic agent.

We have previously shown that PG102 could regulate the expression of IgE and Th1 and Th2 cytokines involved in the allergy pathogenesis, using the OVA-sensitized mouse, NC/Nga dermatitis mouse, magnesium-deficient hairless rat and OVA-induced asthmatic mouse models [5–7]. These in vivo findings were also strongly supported by the data from various in vitro cell culture experiments [5, 7]. All these results suggested that PG102 might have preventive or therapeutic effects on a broad range of allergic diseases. In this study, we tested whether PG102 could prevent allergic diarrhea in a murine OVA-induced allergic diarrhea model. Oral administration with PG102 could sharply decrease the incidence of diarrhea. Our data suggested that PG102 might have suppressed diarrhea incidence by lowering the level of IgE, subsequently inhibiting the infiltration of $CD117^{hi}Fc\varepsilon RI^{hi}$ mast cell to the large intestine (as supported by the flow cytometry analysis of LPLs), and suppressing the inflammation as evidenced by the decreased level of IL-6 and MCP-1 in vitro as well as in vivo.

PG102 highly decreased the level of IL-6, both in vivo and in vitro, while greatly lowering the amount of MCP-1 in isolated splenocytes and, to a lesser extent, in the serum. The magnitude of suppressive effects was similar to that of Dex. IL-6 is deeply involved in fever induction and the acute-phase response, while MCP-1 induces the recruitment of T lymphocytes, monocytes, eosinophils and basophils, generating a series of inflammatory reactions [14, 15, 18, 19]. The fact that PG102 could efficiently downregulate the production of IL-6 and MCP-1 suggested that PG102 might be effectively used to control inflammatory reactions.

The effect of PG102 on IL-6 and IL-10 is interesting in the context of tolerance. IL-6 secreted by dendritic cells, together with unidentified factors produced by myeloid dendritic cells, are thought to interrupt the anergy status of CD4+CD25+ regulatory T (Treg) cells in the spleen [13, 20, 21]. IL-10 plays a key role in the differentiation of naïve CD8 T cells into Tr1 cells, a subset of Treg cells, and the induced Tr1 cells secrete a large amount of IL-10 [22–25]. That is, the tolerance could be inhibited by IL-6 but induced by IL-10, through the regulation of Treg cells. For this reason, it is tantalizing to hypothesize that one mechanism of how PG102 controls food allergies might be via the induction of immunological tolerance.

PG102 is a water-soluble extract prepared from an edible fruit. An active compound has yet to be found, and the underlying mechanism remains to be unraveled. However, several bioassays based on in vitro cell culture systems have been developed. In this study, the effect of PG102 on IL-4 expression was used to prepare PG102 in a consistent manner, using RBL-2H3 cells. The use of such an assay system helps to identify the active compounds and elucidate the molecular mechanisms at molecular and cellular levels as well as to control experimental reagents. Our extensive safety experiments have shown that PG102 is very safe. Together with efficacy data obtained from animal disease models, PG102 demonstrates great potential as a safe and effective reagent for food allergies as well as other allergic diseases, including dermatitis and asthma.

Acknowledgements

We thank Mi-Jung Kim, Eun-Jung Kwon and Mi-Young In for their excellent assistance. This study was funded by grants from the Plant Diversity Research Center of 21C Frontier R&D Programs (Ministry of Education, Science and Technology; M106KD010015-08K0401-01520), the Korean Health 21 R&D Project (Ministry of Health, Welfare and Family Affairs; A050440 and A060655) and the SRC program of KOSEF (R11-2005-009-06003-0).

References

1 Strobel S, Hourihane JO: Gastrointestinal allergy: clinical symptoms and immunological mechanisms. Pediatr Allergy Immunol 2001; 12(suppl 14):43–46.

2 Helm RM: Food allergy animal models: an overview. Ann NY Acad Sci 2002;964:139–150.

3 Seibold F: Food-induced immune responses as origin of bowel disease? Digestion 2005; 71:251–260.

4 Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G: The allergic cascade: review of the most important molecules in the asthmatic lung. Immunol Lett 2007;113:6–18.

5 Park EJ, Kim B, Eo H, Park K, Kim Y, Lee HJ, Son M, Chang YS, Cho SH, Kim S, Jin M: Control of IgE and selective T_H1 and T_H2 cytokines by PG102 isolated from *Actinidia arguta*. J Allergy Clin Immunol 2005;116:1151–1157.

6 Park EJ, Park KC, Eo H, Seo J, Son M, Kim KH, Chang YS, Cho SH, Min KU, Jin M, Kim S: Suppression of spontaneous dermatitis in NC/NGA murine model by PG102 isolated from *Actinidia arguta*. J Invest Dermatol 2007;127:1154–1160.

7 Kim D, Kim SH, Park EJ, Kang CY, Cho SH, Kim S: Anti-allergic effects of PG102, a water-soluble extract prepared from *Actinidia arguta*, in a murine ovalbumin-induced asthma model. Clin Exp Allergy 2009;39: 280–289.

8 Kweon MN, Yamamoto M, Kajiki M, Takahashi I, Kiyono H: Systemically derived large intestinal $CD4^+$ TH2 cells play a central role in STAT6-mediated allergic diarrhea. J Clin Invest 2000;106:199–206.

9 Kweon MN, Fujihashi K, VanCott JL, Higuchi K, Yamamoto M, McGhee JR, Kiyono H: Lack of orally induced systemic unresponsiveness in IFN-γ knockout mice. J Immunol 1998;160:1687–1693.

10 Hirano T, Yamakawa N, Miyajima H, Maeda K, Takai S, Ueda A, Taniguchi O, Hashimoto H, Hirose S, Okumura K, et al: An improved method for the detection of IgE antibody of defined specificity by ELISA using rat monoclonal anti-IgE antibody. J Immunol Methods 1989;119:145–150.

11 Kurashima Y, Kunisawa J, Higuchi M, Gohda M, Ishikawa I, Takayama N, Shimizu M, Kiyono H: Sphingosine 1-phosphate-mediated trafficking of pathogenic TH2 and mast cells for the control of food allergy. J Immunol 2007;179:1577–1585.

12 Sampson HA: Update on food allergy. J Allergy Clin Immunol 2004;113:805–819; quiz 820.

13 Doganci A, Sauer K, Karwot R, Finotto S: Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. Clin Rev Allergy Immunol 2005;28:257–270.

14 Rose CE Jr, Sung SS, Fu SM: Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. Microcirculation 2003;10:273–288.

15 Scheller J, Rose-John S: Interleukin-6 and its receptor: from bench to bedside. Med Microbiol Immunol 2006;195:173–183.

16 Kweon M, Takahashi I, Kiyono H: New insights into mechanism of inflammatory and allergic diseases in mucosal tissues. Digestion 2001;63(suppl 1):1–11.

17 Leung DY, Sampson HA, Yunginger JW, Burks AW Jr, Schneider LC, Wortel CH, Davis FM, Hyun JD, Shanahan WR Jr: Effect of anti-IgE therapy in patients with peanut allergy. N Engl J Med 2003;348:986–993.

18 Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B: Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. FASEB J 1994;8:1055–1060.

19 Jones SA, Novick D, Horiuchi S, Yamamoto N, Szalai AJ, Fuller GM: C-reactive protein: a physiological activator of interleukin 6 receptor shedding. J Exp Med 1999;189:599–604.

20 Detournay O, Mazouz N, Goldman M, Toungouz M: IL-6 produced by type I IFN DC controls IFN-γ production by regulating the suppressive effect of CD4+ CD25+ regulatory T cells. Hum Immunol 2005;66:460–468.

21 Pasare C, Medzhitov R: Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033–1036.

22 Wan YY, Flavell RA: The roles for cytokines in the generation and maintenance of regulatory T cells. Immunol Rev 2006;212:114–130.

23 Akdis M, Blaser K, Akdis CA: T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. J Allergy Clin Immunol 2005;116: 961–968; quiz 969.

24 Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F: An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 1999;190:995–1004.

25 Gilliet M, Liu YJ: Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. J Exp Med 2002;195:695–704.

Curr Allergy Asthma Rep
DOI 10.1007/s11882-010-0097-z

Aberrant Interaction of the Gut Immune System with Environmental Factors in the Development of Food Allergies

Jun Kunisawa · Hiroshi Kiyono

Abstract The gastrointestinal immune system is a major component of the mucosal barrier, which maintains an immunologic homeostasis between the host and the harsh environment of the gut. This homeostasis is achieved by immunologic quiescence, and its dysregulation is thought to result from the development of immune diseases such as food allergies. Recent findings have revealed versatile pathways in the development of intestinal allergies to certain food antigens. In this review, we summarize the regulatory and quiescence mechanisms in the gut immune system and describe aberrant interactions between the host immune system and the gut environment in the development of food allergies.

Keywords Food allergy · Mucosal immunology · Vitamin · Commensal bacteria

Introduction

During the past several decades, the number of people suffering from allergic diseases has increased to the point at which it is a major concern worldwide [1]. Food allergy is a serious disease associated with diarrhea; vomiting; drops in body temperature; weight loss; and, occasionally, life-threatening anaphylactic responses. Aberrant responses to dietary materials are due mainly to type I allergic responses, which are mediated by sequential immune disorders (Fig. 1). Initially, allergen-specific IgE production is induced by the T-helper type 2 (Th2) environment along with dysregulation of regulatory immune responses, which promote mast cell infiltration into the intestine. Subsequently, secondary cross-linking by the allergen on mast cells via Fcε receptor results in the production of various allergic mediators by mast cells (eg, histamine, platelet-activating factor, leukotrienes, and mast cell protease-1). These mediators increase intestinal permeability, exacerbating the allergic symptoms [2].

Although classic immediate food allergies are mediated by mast cells, food allergens lead to the induction of delayed or chronic allergic reactions as well. The mechanisms underlying these delayed reactions are not fully understood but are thought to involve the accumulation of eosinophils in the gut (Fig. 1) [3]. A pathogenic mediator, major basic protein, was detected in the accumulated place of eosinophils in the gut, causing gut tissue damage and associated symptoms, including diarrhea, bloody stools, and blood eosinophilia [3].

In spite of continual ingestion of the same dietary materials, many people show no aberrant reactions to allergens. This unresponsiveness is associated with an immunologic tolerance known as oral tolerance, which involves the specific suppression of cellular and humoral immune responses to ingested antigens [4]. Several lines of evidence indicate that oral tolerance is achieved by a unique gut immune system made up of complex regulatory networks among immunocompetent cells (eg, dendritic cells [DCs] and T cells) [5]. The establishment of food allergy models using experimental animals allows the investigation of possible pathways involved in the abrogation of the immunologic regulatory network and the consequent development of food allergies [6•]. It also allows the identification of some immunologic characteristics as they appear in human patients, revealing basic

---

J. Kunisawa · H. Kiyono (✉)
Division of Mucosal Immunology, Institute of Medical Science,
The University of Tokyo,
Tokyo 108-8639, Japan
e-mail: kiyono@ims.u-tokyo.ac.jp

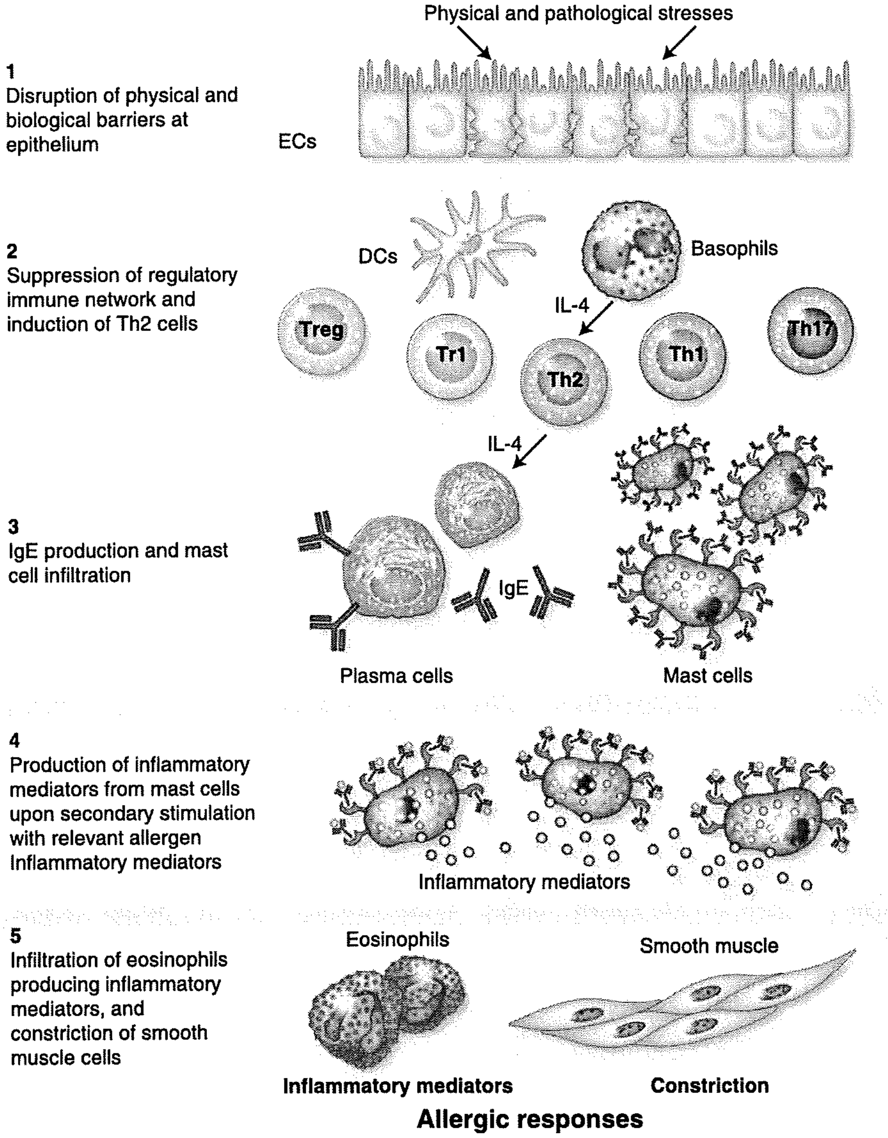


Fig. 1 Multiple steps in the development of allergic responses. **1**, Several stresses, including psychological, bacterial, and cytokine stimulation, disrupt the epithelial barrier, permitting the penetration of allergens. **2**, The immunologic environment mediated by dendritic cells (DCs) and presumably basophils results in the preferential induction of T-helper type 2 (Th2) cells, which leads to **3**, the induction of IgE production and mast cell infiltration. **4**, Mast cells produce inflammatory mediators (eg, histamine, prostaglandins, and leukotrienes) upon cross-linking of IgE with the allergen, leading to **5**, the constriction of smooth muscle cells and the recruitment of eosinophils. EC—epithelial cell; IL—interleukin; Treg—regulatory T cell

aspects of allergic responses and potential clinical targets against food allergies.

Accumulating evidence indicates that environmental factors in the gut (eg, commensal bacteria) play an important role in maintenance and disruption of gut immune quiescence [7]. Indeed, previous studies using germ-free mice showed that stimulation by commensal bacteria promotes the development of active and quiescent immune responses [8]. Recent advances in genome-based bacterial analyses have revealed quantitative and qualitative aspects of commensal bacteria, including unculturable bacteria, in the development and dysregulation of the host immune system [9]. Other recent nutritional studies have indicated that diversification in food, particularly Westernized diets, may be associated with the increased number of allergic patients [1].

In this review, we focus on the gut immune system in the development of food allergies from the viewpoint of the quiescent immune system and cross-talk with environmental factors.

Gut Regulatory Immune Networks and Their Disruption in the Development of Food Allergies

The gut immune system is a unique system that can distinguish between harmless and harmful nonself materials [10]. Accumulating evidence shows that various immunocompetent cells participating in different gut immune responses, including physical, innate, and acquired immunity, use immunologic cross-talk to negatively regulate the immune responses to harmless materials. The tight junction among epithelial cells (ECs) is an example of a physical barrier that prevents the uptake of allergenic materials. Disruption of epithelial barriers promotes the development of food allergies: psychological stress [11], bacterial infection (eg, by *Candida albicans*) [12], and cytokine stimulation (eg, by IL-9) [13••] resulted in the increased permeability of epithelial layers, which increased the susceptibility to allergens. Similarly, immature development of the epithelial barrier in infants may explain the prevalence of food allergies in infants younger than 3 years old [1]. Additionally, ECs are not simply a physical barrier; they also influence the biological nature of allergenic macromolecules through the production, formation, and synthesis of secretory IgA and digestive enzymes. Thus, ECs pose physical, physiologic, and immunologic barriers to allergenic materials.

At the T-cell level, the classic paradigm is that Th2 responses favor the development of allergic responses, whereas Th1 responses inhibit them [14]. In this context, our group reported that the homodimeric form of interleukin (IL)-12 p40 (p80) is produced predominantly in the large intestine of allergic mice and plays an important role in the induction of Th2 responses by competing with heterodimeric IL-12 (p40 + p35), an essential cytokine for the induction of Th1 responses (Fig. 2) [15]. Although it is not clear which kinds of cells are responsible for the IL-12 p80 production, it could be worthwhile to examine basophils as immunoregulatory antigen-presenting cells involved in the process of inducing an aberrant Th2-type environment. Recent reports show that basophils express major histocompatibility complex class II and costimulatory molecules (eg, CD80 and CD86) together with the predominant production of IL-4, initiating Th2 responses (Fig. 2) [16••, 17, 18]. Surprisingly, DCs are not required for the induction of Th2 responses; basophils alone are sufficient. Although the role of basophils in the development of food allergies has not yet been tested, this is an important point to be investigated.

The development of allergic responses is not explained simply by the classic Th1/Th2 paradigm. Current attention is focused on the regulatory T-cell (Treg) network. This network, composed of Treg, Tr1, Th3, and CD8αα T cells, plays a key role in the achievement of immunologic quiescence (Fig. 2) [19, 20]. Tregs are abundant in the intestinal compartments for the creation of immunologic quiescent conditions in their harsh environments. As Tregs developing naturally in the thymus, de novo-generated intestinal Tregs express forkhead box P3 (FoxP3), a master transcription factor for the differentiation of Tregs, and have been implicated in the negative regulation of allergic responses [21, 22•]. The de novo differentiation of Tregs from naïve CD4 T cells requires transforming growth factor (TGF)-β, a cytokine that is abundant in the intestine. Importantly, costimulation with IL-6 plus TGF-β leads to the exclusive induction of IL-17–producing T (Th17) cells, which are involved in the induction and inhibition of inflammatory and allergic diseases (Fig. 2) [23-25]. Reciprocally, all-trans retinoic acid (at-RA), a metabolite of vitamin A produced particularly by intestinal CD103$^+$ DCs, prevented the differentiation of Th17 cells but enhanced Treg induction in the intestine (Fig. 2) [26••, 27-29]. It was reported recently that ECs educate intestinal CD103$^+$ DCs to be tolerogenic through the production of TGF-β and at-RA (Fig. 2) [30•]. Additionally, Tregs reciprocally educate DCs to produce IL-27 for the subsequent induction of Tr1 cells, a distinct Treg population (Fig. 2) [31••]. Like Tregs, Tr1 cells produce IL-10, but unlike Tregs, they do not express FoxP3. These data suggest that the cytokine milieu created by T cells, DCs, ECs, and basophils is critical for the creation and maintenance of immunologic homeostasis in the gut. Further molecular and cellular investigation of this intestinal regulatory system is required for the development of new immunotherapy for food allergies.

Commensal Bacteria in the Regulation of the Gut Immune System

Because the prevalence of food allergies has increased very rapidly in industrialized countries, environmental and host factors are considered to be involved. Among several environmental factors, commensal bacteria are likely to be pivotal in the regulation of the gut immune system because they initiate their intestinal habitation at birth and continuously grow and are required for the maturation of the gut immune system, including the induction of oral tolerance [32]. This idea, known as the *hygiene hypothesis*, suggests that the improvement of hygiene, the development of antibiotics and vaccines, and the intake of almost-sterile food have reduced the gut's exposure to microorganisms and thus have led to the failure of the maturation of the gut immune system [7]. The hygiene hypothesis is supported by several epidemiologic studies, although the issue is still controversial [7]. Supporting the hypothesis, it was reported that mice lacking Toll-like receptor 4 (TLR4), a receptor for lipopolysaccharide, showed high susceptibility to food allergy [33], suggesting that signals dependent on innate immunity influence the allergic responses. Allergic TLR4-deficient mice showed Th2-biased responses in intestinal and systemic (eg, spleen) compartments. This finding correlated with another finding that a defect in MyD88,

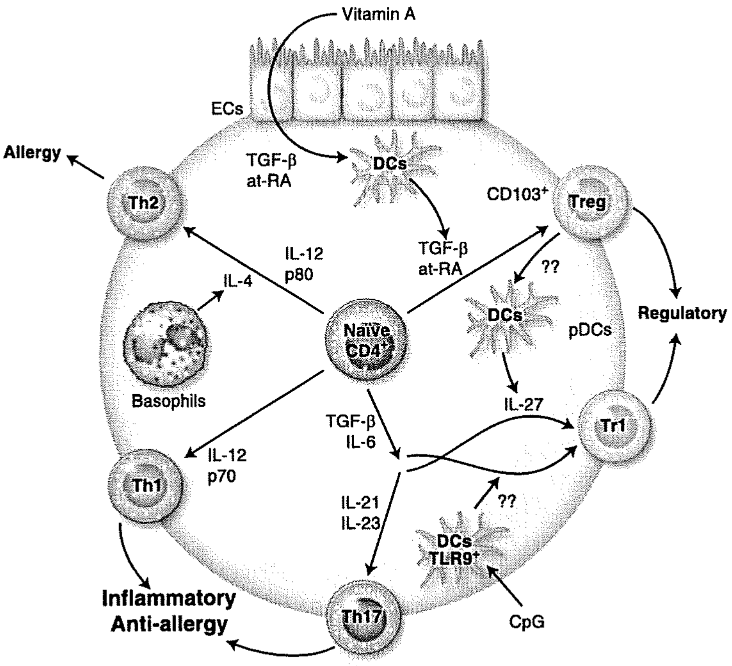


Fig. 2 Versatile pathways for the induction of regulatory and pathological T-cell network. Epithelial cells (ECs) produce transforming growth factor (TGF)-β and all-trans retinoic acid (at-RA), which make CD103$^+$ dendritic cells (DCs) tolerogenic. Naïve CD4$^+$ T cells activated by CD103$^+$ DCs differentiate into regulatory T cells (Tregs) also via at-RA and TGF-β. Tregs subsequently educate plasmacytoid DCs (pDCs) to produce interleukin (IL)-27, which is required for the induction of IL-10–producing Tr1 cells, another type of Treg. Tr1 cells are also induced by CpG-treated DCs. On the other hand, IL-23 and IL-12 p70 are involved in the induction of T-helper type 17 (Th17) and Th1 cells, respectively. Th2 cells, a major T-cell population in the development of allergic responses, require IL-4, which is predictably produced by basophils. TLR—Toll-like receptor

an adopter molecule for many TLRs, moved the T-cell responses toward the Th2 type [34]. Reciprocally, stimulation with DNA-containing unmethylated CpG induces Th1-type immune responses via TLR9 [33]. In addition to Th1-type immune responses, a TLR9-mediated signal is a prerequisite for the efficient induction of regulatory-type T cells (eg, Tregs and Tr1 cells). Indeed, oral administration of a TLR9 agonist inhibited the development of allergic responses to peanuts [33]. In this context, a recent study revealed a reciprocal relationship between retinoic acid and TLR9-mediated signals in the induction of Tregs [35••]. As mentioned previously, costimulation of CD4 T cells with at-RA enhances TGF-β–mediated FoxP3 expression; however, at-RA inhibits IL-10 induction [35••]. On the other hand, stimulation of DCs via TLR9 reduces FoxP3 expression and upregulates IL-10 induction in CD4 T cells (Fig. 2). Although the physiologic roles of the reciprocal regulation systems via at-RA and TLR9 in the development of food allergies are still unclear, these reports reveal a multilayered system involved in the negative regulation of antigen (or allergen)-specific immune responses in the harsh environment of the gastrointestinal tract.

In addition to hematopoietic cells (eg, T cells and DCs), ECs also express various kinds of TLRs [36]. For instance, the tight junction between ECs is enhanced by a TLR2-mediated signal, indicating that bacterial stimulation is required for the first physical barrier to prevent the penetration of allergens as almost intact protein [37]. In addition to TLR2, TLR9 is a potential innate receptor in the regulation of EC function. TLR9 recognizes unmethylated CpG-containing bacterial DNA and is expressed on the apical and basolateral surfaces of ECs [36]. Intriguingly, TLR9 stimulation at the apical site activates nuclear factor-κB without the production of inflammatory cytokines, whereas basolateral stimulation of TLR9 results in the robust production of inflammatory cytokines [38].

In line with the hygiene hypothesis, probiotic bacteria are used to prevent allergic diseases [39]. Although the precise mechanisms used by probiotics to prevent and treat allergies are not fully understood, several pathways are considered possible mechanisms. In addition to imposing a physical barrier to compete with pathogenic bacteria, probiotics directly stimulate the immune system to establish a regulatory network, particularly in the induction of inhibitory cytokines (eg, IL-10) [40]. Furthermore, probiotics contribute indirectly to the regulation of the immune system by producing immunomodulatory molecules through the consumption of foodstuffs. For instance, probiotic bacteria digest exogenous and endogenous materials (eg, fibers and mucins), and the broken down products affect the host immune system [40]. A recent study reported that short-chain fatty acids produced from fiber by commensal bacteria are required for the normal resolution of inflammatory responses through G-protein–coupled receptor 43 [41].

Although many bacteria universally produce various TLR ligands (eg, lipopolysaccharide and CpG-motif DNA) and consume dietary materials, not all bacteria can establish regulatory networks in the gastrointestinal tract. Instead, some commensal bacteria induce inflammatory cells. For instance, recent studies have shown that segmented filamentous bacteria preferentially induce Th17 cells, not Tregs [42, 43]. In line with these findings, it was reported that exogenous adenosine triphosphate derived from commensal bacteria induced Th17 cells [44]. *Lactobacillus* and *Bifidobacterium* are used in the probiotic treatment of allergic diseases on the basis that allergic patients have decreased counts of both [39]. However, among several species of each, only some strains have strong potential as probiotic bacteria. Therefore, the key functions that determine probiotic ability must be determined.

Dietary Materials and Milk in the Development of Food Allergy

The gastrointestinal tissues are vital for the digestion and absorption of nutrients. Because allergic diseases are prevalent in Westernized countries, interactions between dietary factors abundant in Western food and the gut immune system could be involved in the development of food allergies [1]. Among dietary factors, considerable evidence indicates that dietary lipids directly regulate allergic responses, especially omega-3 (eg., linolenic acid) and omega-6 (eg, linoleic acid) fatty acid [45]. Mammals must ingest both forms of these essential fatty acids. Some inflammatory lipid mediators (eg, prostaglandins and leukotrienes) are derived from omega-6 fatty acids, whereas anti-inflammatory mediators (eg, eicosapentaenoic acid and docosahexaenoic acid) are generated from linolenic acid. Thus, the balance between omega-6 and omega-3 fatty acids in dietary oils seems critical to the development of allergic diseases [45]. In support of this notion, clinical studies have shown that omega-3 dietary supplementation or frequent consumption of fish containing abundant omega-3 fatty acids decreases the risk of allergic diseases [46].

Our group showed an immunologic function of another lipid mediator, sphingosine 1-phosphate (S1P), in the development of food allergy [47]. S1P is generated from sphingomyelin and ceramide and regulates cell trafficking through interactions with its receptors [48]. On the basis of our findings on S1P function in the regulation of the gut immune system [49, 50], we suspect that cell trafficking of pathogenic cells (eg, activated pathological T and mast cells) is also regulated by S1P. In fact, treatment of an experimental animal model with an S1P inhibitor resulted in the inhibition of allergic diarrhea, which is associated with decreased accumulation of pathogenic T and mast cells in the large intestine, without affecting serum IgE production [47]. Because it is possible that S1P precursors are present in dietary oils, these oils could be additional factors in the determination of allergic diseases.

Milk is the major dietary material for neonates. Previously, breast milk was thought to be responsible for the allergic responses in neonates as a source of allergens; however, several studies demonstrated that removing allergens from the diet during pregnancy and lactation did not prevent allergies [51]. On the other hand, recent evidence has revealed that breast milk contains molecules that induce tolerance, including IL-10, TGF-β, and immunoglobulins [51]. In agreement with this idea, mouse pups suckled by allergen-exposed mothers showed tolerance to those allergens [52••, 53]. A recent study showed that feeding of breast milk induced tolerance that was dependent on TGF-β but was not dependent on the transfer of immunoglobulins or IL-10 [52••]. The nucleus and biological nature of dietary materials, including lipids and milk, may provide us with new candidate regulatory molecule(s) that can mimic the mucosal Treg cell network system.

Conclusions

Progress in our understanding of immunologic tolerance and its abolition in the development of food allergies suggests several strategies against food allergies [54]. One is the re-education of the disordered gut immune system to induce oral tolerance. Although the prevention of food allergies still requires the prolonged elimination of the allergenic diet, several studies have already achieved immune therapy to prevent food allergy. Immunologic homeostasis between the host immune system and the gut environment is maintained by complex pathways. In particular, interactions among host immunocompetent cells (eg, T cells, DCs, ECs, and basophils) and immunologic modification via dietary materials (eg, vitamin A and short-chain fatty acids) and bacterial products (eg, CpG and adenosine triphosphate) are critical events for the formation and maintenance of immunologic quiescence, and their dysregulation leads to the development of food allergies. Further studies of immunologic cross-talk with gut environments are needed to develop novel strategies for the prevention and treatment of food allergies.