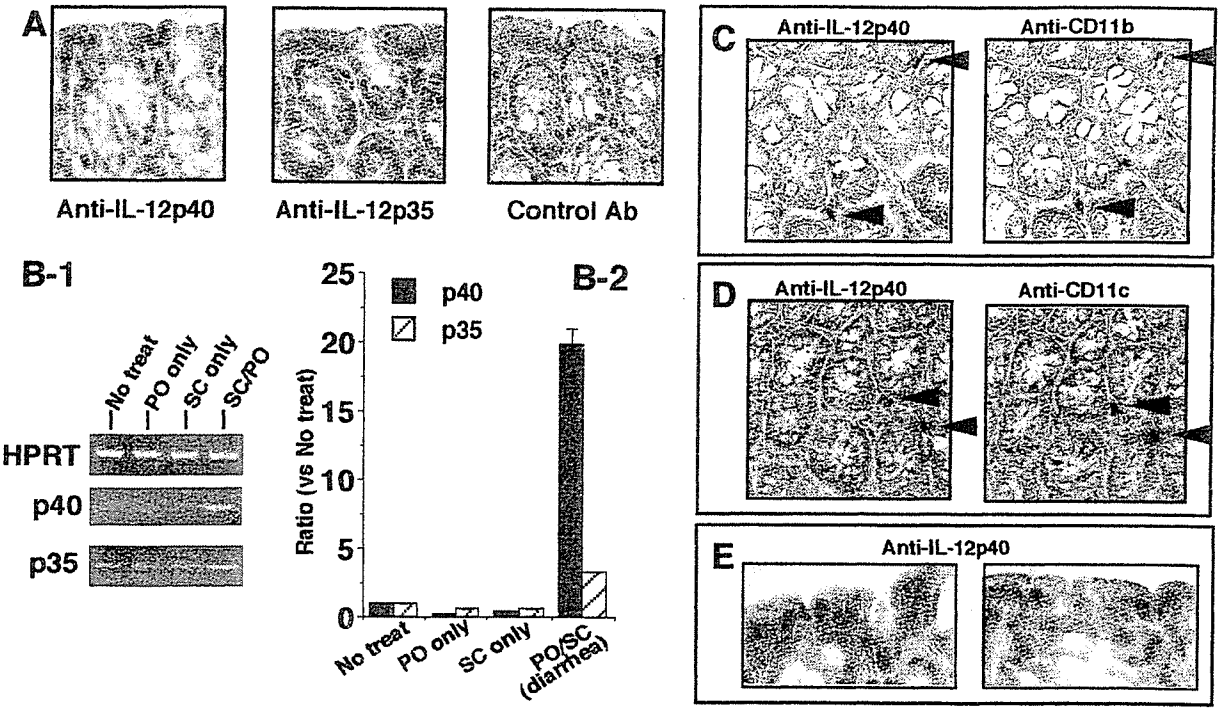

Figure 1. Selective production of IL-12p40 by the large intestine of diarrhea-induced mice. In **A**, large intestinal tissues from diarrhea-induced mice were immunostained with anti-IL-12p40 mAb, anti-IL-12p35 mAb, or control IgG. Control non-disease mice section gave no signal above background (data not shown). In **B-1**, IL-12p40-specific mRNA was expressed selectively in the large intestine of mice with allergic diarrhea. In **B-2**, quantitive real-time PCR analysis of IL-12p40- and p35-specific mRNA expression was performed. The ratio was obtained as the level of IL-12p40 or p35 expression in non-treated mice as a scale of one. The detailed information for the expression of this ratio is described in the Materials and Methods section. In **C-E**, IL-12p40 was detected in MØ and DC and epithelial cells in the large intestine. The serial sections of the large intestine from diarrhea-induced mice were stained with anti-IL-12p40 mAb and anti-CD11b mAb (**C**), with anti-IL-12p40 mAb and anti-CD11c mAb (**D**). The **arrows** point to double-positive cells. Large intestinal epithelial cells were stained with anti-IL-12p40 mAb (**E**).

body and ABC-AP. The color reaction was developed using Vector Red Substrate Kit I.

Statistical Analysis

Statistical analyses were performed by the two sample non-parametric Welch test with a significance level of 0.01 (**) for body weight and Ig levels, respectively. Mouse disease rates were determined using the Wilcoxon rank-sum test with a significance level of 0.01 (**). Values for cytokine-synthesis in the samples between anti-IL-12 p40-treated and control antibody-treated mice were analyzed by using Student's *t*-test at *P* values of <0.01(**).

Results

Detection of IL-12p40 Protein in the Large Intestine of Allergic Diarrhea Mice

To examine whether IL-12p40 was expressed in the large intestine of OVA-induced diarrhea mice, we analyzed IL-12 expression using a variety of available detection methods. First, we performed immunohistochemical analysis to directly demonstrate the enhanced IL-12p40 expression in the large intestine of mice with allergic diarrhea. As shown in Figure 1A, IL-12p40, but not IL-12p35, was expressed in the large intestine of diarrhea-induced mice. To further confirm enhanced expression of IL-12p40 in the large intestine of mice with diarrhea, we next performed IL-12-specific RT-PCR analysis. Interestingly, IL-12p40 mRNA was only detected in the large intestine of diarrhea-induced mice, not in control mice without the disease [eg, SC only or per oral challenge (PO) only; Figure 1B]. In contrast, IL-12p35 mRNA expression was detected in both groups of mice (Figure 1B-1). When IL-12-specific mRNA quantitative real-time PCR analysis was performed, high levels of IL-12p40-specific mRNA were noted in the large intestine of OVA-induced allergic diarrhea mice (Figure 1B-2). In contrast, the level of p35 did not vary among the four different groups including experimental diseased (SC/PO) and control non-diseased mice (non-treated, SC only, and PO only). Taken together, these results clearly indicate that IL-12p40, but not p35, was selectively enhanced at the levels of both mRNA and protein in the large intestine of allergic diarrhea mice.

Inasmuch as the induction of IL-12p40 selectively occurred in the large intestine of OVA-induced allergic diarrhea mice, it was important to determine which cell types produced IL-12p40 in the large intestine. Immunohistochemical analysis demonstrated that IL-12p40-producing cells were co-stained with anti-CD11b mAb [ie, macrophages (MØ)]. Further, $CD11c^+$ cells [ie, dendritic cells (DC)] were also positively stained for IL-12p40 (Figure 1, C and D). Further, some epithelial cells were also positive for IL-12p40 expression (Figure 1E). Taken together, these findings show that large intestinal macrophages, dendritic cells, and epithelial cells are responsi-

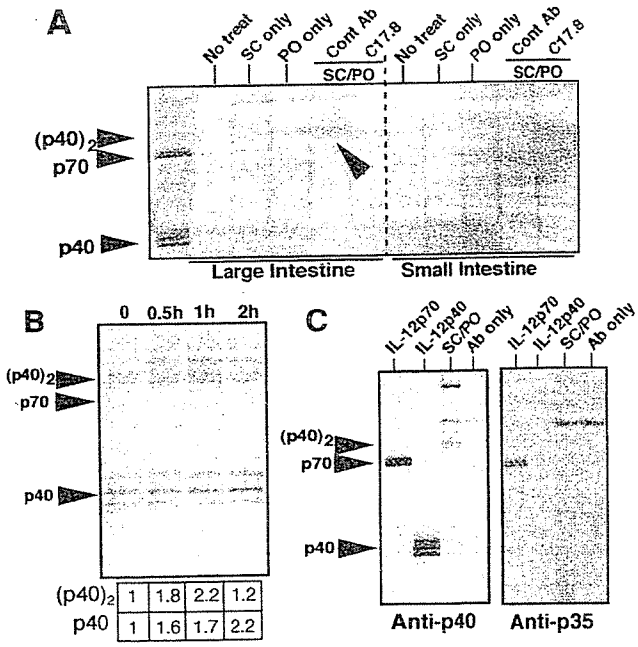

Figure 2. Induction of IL-12p40 homodimer in the large but not small intestine of diarrhea-induced mice. Large and small intestinal tissue extracts were subjected to immunopreciptation and Western blotting analysis using anti-IL-12p40 (C17.8) mAb under non-reducing conditions (**A**). The captions above the figure indicate the experimental mouse group receiving different *in vivo* treatments. Thus, the samples were obtained from SC/PO mice treated with C17.8 or control antibodies. Further, the samples were isolated from mice treated with PO only, SC only, or non-treated mice. The **arrow** points to IL-12p40 homodimer expression in the large intestine of diarrhea-induced mice. The data represent four independent experiments. In **B**, at the indicated times after oral administration of OVA, large intestinal tissue extracts isolated from diarrhea-induced mice were assayed for IL-12p40 by the same method as in **A**. In **C**, the large intestinal tissue extracts of diarrhea-induced mice were subjected to Western blotting with anti-IL-12p35 Ab as well as anti-IL-12p40. IL-12p70 protein was used as a positive control for the IL-12p35 detection system. As negative control, immunoprecipitation was performed without the tissue specimens (Ab only). The data represent three different experiments.

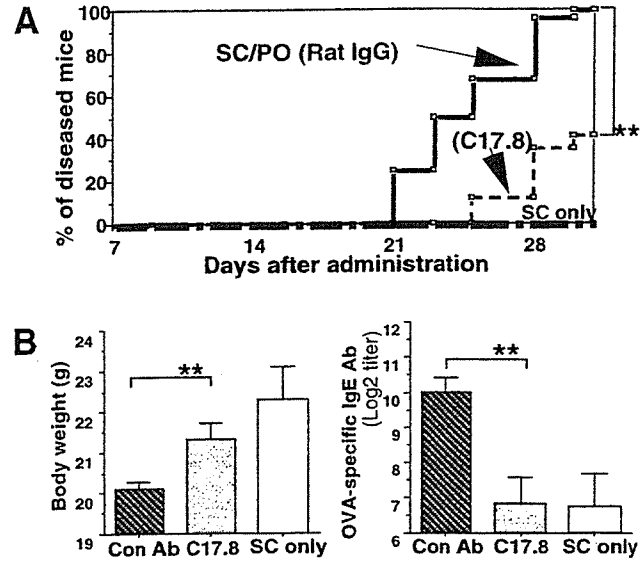

Figure 3. Inhibition of allergic diarrhea disease by the treatment with anti-IL-12p40 mAb. In **A**, anti-IL-12p40 mAb (C17.8) treatment (**thin dashed line**) delayed the development of allergic diarrhea when compared with the rat IgG-treated group (**solid line**). Statistical differences were determined by Wilcoxon rank-sum test and are indicated by **, $P < 0.01$. Mice with SC only were used as controls (**thick dashed line**). In **B**, **left**, body weight was recovered in allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). In **B**, **right**, OVA-specific IgE Abs were reduced in the serum of allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). The data are expressed as the mean of ± SE and are representative of five independent experiments. Statistical differences between anti-IL-12p40 mAb and control rat IgG-treated mice are indicated as **, $P < 0.01$.

ble for the production of IL-12p40 at the disease site of OVA-induced allergic diarrhea.

The Western blotting method was adopted for the examination of IL-12 p40 expression in the small and large intestinal tissue extracts from OVA-induced allergic diarrhea mice within 1 to 2 hours after the last oral challenge. In the large intestine of diarrhea-induced mice, the 80kD form of IL-12 predominated clearly demonstrating the presence of IL-12p40 homodimer but not 70kD IL-12 heterodimer, in contrast to the environment observed in the large intestine of control mice or the small intestine of mice with/without diarrhea (Figure 2A). The multiple bands of p40 and p80 are the result of glycosylation heterogenity.[15] We thus analyzed three bands of p40 and three bands of p80 as specific bands. In the case of spleen, IL-12p40 was detected in control, healthy mice. The levels of IL-12p40 did not change after development of allergic diarrhea (data not shown). To examine the kinetics of the response, we next assessed the time course of IL-12p40 expression in the large intestine of the diarrhea-induced mice. The expression of IL-12p40 or p80 in the large intestine peaked between 1 and 2 hours after the last oral challenge, at the same time that severe symptoms of OVA-induced allergic diarrhea were observed (Figure 2B). These data suggest that there is an intimate relationship between the development of diarrhea and the expression of IL-12p40 in the large intestine.

To further confirm the expression of IL-12p80 or p40 instead of the p70 form, the protein extracts from the large intestine of the diarrhea-induced mice were immunoprecipitated with anti-IL-12p40 mAb and then Western blotting was performed using anti-IL-12p35 mAb. No molecular bands corresponding to IL-12p70 proteins were detected in the large intestine of diarrhea-induced mice, while predominant IL-12p40 protein was detected (Figure 2C). The large molecular weight band above the p70 and p80 bands was non-specific and was caused by the nature of antibody used in the immunoprecipitation, since the large molecular weight band was also seen following immunoprecipitation in the absence of tissue specimens (Ab only in Figure 2C). These results indicate that the secretion of IL-12p40, but not IL-12p70, in the large intestine is critically important in the development of OVA-induced allergic diarrhea.

Anti-IL-12p40 Treatment Reduced the Symptoms of Allergic Diarrhea

Inasmuch as the preferential localization of IL-12p40 was observed in mice with allergic diarrhea, we next performed a neutralization experiment using anti-IL-12p40 mAb (C17.8). We observed a significant delay in the onset of diarrhea and reduced the frequency of diarrhea to 40% by treatment with anti-IL-12p40 mAb (Figure 3A). Obvious body weight loss was seen in control Ig-treated

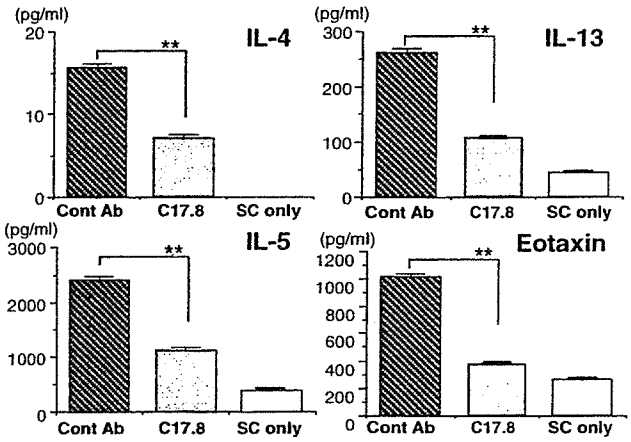

Figure 4. *In vivo* treatment with anti-IL-12p40 (C17.8) reduced the predominant antigen-specific Th2 type responses by large intestinal mononuclear cells isolated from diarrhea-induced mice. The mononuclear cells isolated from the large intestine (1.5×10^5 cells/well) were cultured with OVA (1 mg/ml) for 3 days. Culture supernatants were harvested and then assayed for IL-4, IL-13, IL-5, and eotaxin by ELISA assay. These data are expressed as the mean ± SE and are representative of three independent experiments. The statistical differences between anti-IL-12p40 mAb and control antibody treated mice are indicated as **, $P < 0.01$.

diarrhea mice, while treatment with anti-IL-12p40 mAb resulted in partial recovery from body weight loss (Figure 3B, left). In addition, high levels of OVA-specific IgE Abs were detected in the serum of diarrhea-induced mice treated with control Ab, whereas the mice treated with anti-IL-12p40 mAb showed low levels of OVA-specific IgE Abs (Figure 3B, right). These results indicate that treatment with anti-IL-12p40 mAb alters the environment from a disease-inducing one to one fastening recovery in OVA-induced allergic diarrhea.

Suppression of Intestinal Th2-Type Cytokine by Anti-IL-12p40 Treatment

To confirm decreased Th2-type responses in the large intestine after anti-IL-12p40 mAb treatment, we next examined antigen-induced cytokine production by the large intestinal mononuclear cells. Interestingly, the anti-IL-12p40 treatment resulted in decreased levels of OVA-induced Th2 cytokine synthesis including those of IL-4, IL-5, and IL-13 (Figure 4). Production levels of the Th2 cytokines were comparable to those of control mice without allergic diarrhea (SC only). In contrast to the alterations observed in OVA-induced Th2 cytokine synthesis, there was no difference in the level of IFN-γ production between the mice treated with anti-IL-12p40 mAb and control IgG (data not shown). We further confirmed that IL-4 producing cells were $CD4^+$ Th2 cells by intracellular staining (data not shown).

Finally, the level of eotaxin, a well-known chemokine for eosinophil recruitment in allergic disease,[30] was also examined, since our previous study demonstrated that the frequency of eosinophils was increased in the large intestine of allergic diarrhea mice.[1] Likewise, the level of eotaxin could also be presumed to be increased in the large intestine of allergic diarrhea mice (Figure 4). Interestingly however, the level of eotaxin synthesis was significantly decreased by the treatment with anti-IL-12p40 mAb (Figure 4). These results indicate that anti-IL-12p40 mAb inhibited the immunopathological Th2 cytokine environment of the large intestine in allergic diarrhea mice. Thus, an interesting scenario could be the presence of high levels of IL-12p40 monomer and/or homodimers instead of IL-12p70 in the disease site of OVA-induced allergic diarrhea mice. Therefore, treatment with anti-IL-12p40 mAb might result in the inhibition of Th2-type responses in the large intestine of allergic disease mice.

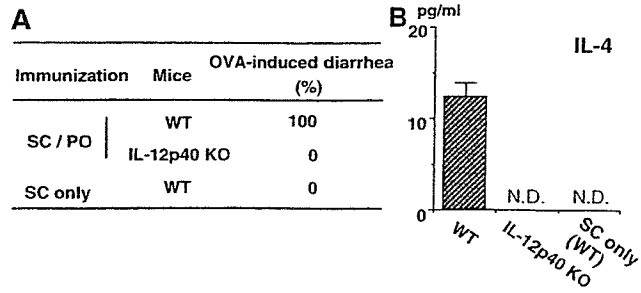

Figure 5. Suppression of allergic diarrhea development in IL-12p40 KO mice. In **A**, the incidence of allergic diarrhea was reduced in the IL-12p40 KO mice when compared with wild-type mice immunized subcutaneously and then given OVA repeatedly by the oral route (SC/PO). In **B**, the large intestinal LP mononuclear cells from IL-12p40 KO mice did not produce IL-4. Mononuclear cells isolated from the large intestine were restimulated with OVA for the assessment of IL-4 synthesis as described in Figure 4A. The data are expressed as the mean ± SE and represent three different experiments.

IL-12p40-Deficient Mice Do Not Develop Allergic Diarrhea

To directly confirm the pathological role of IL-12p40 in the development of allergic diarrhea, IL-12p40 knockout (KO) mice were used. IL-12p40 KO mice did not develop the allergic diarrhea completely (Figure 5A). OVA-induced IL-4 production by large intestinal LP mononuclear cells was not detected in IL-12p40 mice (Figure 5B). The levels of other Th2-type cytokines (IL-5 and IL-10) were also reduced in IL-12p40 KO mice (data not shown). Taken together, these results clearly show that IL-12p40 plays an important role in the development of this large intestinal allergic disease.

Discussion

Our present findings provide new, strong evidence for an immunopathological role for locally produced IL-12p40 in the development of OVA-induced allergic diarrhea. Here we demonstrate the high expression of IL-12p40, without IL-12p35, in the large intestine but not in the small intestine of mice with allergic diarrhea. These IL-12p40 were locally produced by large intestinal MØ, DC, and epithelial cells. Based on our knowledge, this is the first demonstration of the presence of IL-12p40 in the selected part of the intestinal tract (eg, large intestine) in mice with allergic diarrhea. Although IL-12 is thought to drive the Th1-dominant environment,[31] our present findings provide additional supportive evidence that IL-12p40 contributes to the generation of a Th2-dominant environ-

ment.[18,19] It should be noted that our results directly demonstrate the *in vivo* immunopathological contribution of locally produced mucosal IL-12p40 to the development of OVA-induced diarrhea. Thus, the anti-IL-12p40 treatment reduced the incidence of OVA-induced allergic diarrhea. An attractive explanation would be that large intestinal MØs and DCs as well as epithelial cells contribute to the development of pathological Th2-dominant responses by the production of IL-12p40 in OVA-induced allergic diarrhea. Thus, the administration of anti-IL-12p40 resulted in the inhibition of the locally produced, mucosal IL-12p40-created, pathological Th2 condition, leading to the reduction of disease development.

Our present and previous results clearly show that large intestinal antigen-specific Th cells produce high levels of Th2 cytokine in OVA-induced allergic diarrhea.[1] The presence of monomeric or dimeric forms of IL-12p40, behaving as an antagonist to IL-12p70, is an additional contributing factor for the creation of a dominant pathological Th2 environment. Thus, the severe symptoms of allergic diarrhea were reduced by treatment with anti-IL-12p40 mAb, since the production of Th2 cytokines was significantly decreased in the large intestine. Overall, IL-12p40-supported, Th2-type cytokine synthesis plays a critical and pathological role in the induction of allergic reactions in large intestinal tissues. Although we do not have any specific explanation for the generation of IL-12p40 at the disease site, one possibility could be antigen overload in the intestinal tract. Our previous study demonstrated that oral administration of high doses of OVA induced Th2-mediated allergic diarrhea in systematically pre-sensitized BALB/c mice.[1] In contrast, low doses of oral OVA failed to induce allergic diarrhea. It was also shown that high doses of OVA peptide increased the numbers of naive $CD4^+$ T cells with Th2-like phenotype, which in turn produced dramatically large amounts of IL-4.[32] Therefore, high doses of oral antigen may create an immunological environment favoring Th2 cell development. To support this view, it has also been shown that high doses of oral antigen preferentially inhibit IFN-γ-producing Th1-type cells.[1] Further, the dose of antigen can determine whether Th1- or Th2-type cells are generated by antigen-presenting cells including DC.[33] Taken together, these findings allow us to postulate that an overload of oral antigen may direct mucosal antigen-presenting cells, including DC and MØ, and epithelial cells, to produce monomeric or dimeric forms of IL-12p40 instead of IL-12p70.

IL-12 has been considered as an inhibitory factor for allergic responses induced by preferential Th2-cytokine production. Indeed, endogenous rIL-12 decreased IgE levels and Th2 cytokine production induced by allergic reaction.[34] In contrast, IL-12 has also been shown to be involved in the pathological phase of mucosa-associated allergic diseases of the respiratory tract. In the murine asthma model, IL-12 contributed to the recruitment of eosinophils into the respiratory tract via the induction of VCAM-1 on local vascular epithelial cells.[35] Thus, the deletion of the IL-12 gene (p40) resulted in a substantial reduction in the airway recruitment of eosinophils and in the expression of VCAM-1 when compared with wild-type mice exhibiting an asthma-like reaction induced by systemic sensitization followed by nasal OVA.[36] In addition, selective overexpression of IL-12p40 was noted in airway epithelial cells and bronchoalveolar lavage fluids of patients with asthma.[20] Our present findings also demonstrate that the locally produced p40 form of IL-12 was associated with the development of OVA-induced allergic diarrhea. Thus, IL-12p40 was preferentially expressed only in the large intestine of allergic diarrhea mice. In addition to these results generated through the characterization of an asthma model, our present finding suggests a critical role for IL-12, especially that of p40-associated molecules, for the development of allergic diseases including asthma and food allergy.

The treatment with anti-IL-12p40 mAbs effectively reduced the incidence as well as the severity of allergic diarrhea, an effect most likely due to an alteration in the dominant immunopathological Th2-type response to a Th1-type environment. To support this view, locally overexpressed IL-12p40 may compete with the well-known Th1 promoter IL-12p70 and IL-23 (p40/p19).[37–39] To this end, it has been shown that endogenous IL-12p40 can overcome the Th1-promoting activity of IL-12p70 and/or IL-23.[18] In this regard, our recent and separate study showed that IL-23p19-specific mRNA expression was not detected in either diseased or healthy BALB/c mouse groups (data not shown). The results suggest that IL-23 dose not play an important role in the development of our diarrhea model. Therefore, treatment with anti-IL-12p40 antibody likely eliminated the antagonistic effect of IL-12p40 at the local site, perhaps leading to the creation of an IL-12p70 environment for the initiation of down-regulation of Th2 responses. An alternative explanation would be that anti-IL-12p40 mAb used in this experiment may possess a higher affinity for the monomeric or dimeric form of IL-12 than for the IL-12p70 heterodimer. Although our emphasis has been on the inhibitory effects of anti-IL-12p40 mAb for the prevention of allergic diarrhea, one must accept the fact that complete prevention of disease development was never achieved through use of mAbs. A possible explanation for this finding could be that anti-IL-12p40 mAb inhibited Th1 induction of IL-12p70 in addition to IL-12p40. Thus, this alteration of a Th2 dominant environment and shift to one of a Th1-type may partially occur in the large intestine of mice with allergic diarrhea. To support this possibility, the mAb used in these experiments has been shown to neutralize IL-12p70 in addition to IL-12p40.[13,14] In addition, the experiments using IL-12p40 KO mice suggest that the absence of IL-12p40 results in a complete failure to develop allergic diarrhea. It clearly shows that IL-12p40 play a critical role in the development of this disease. However, one alternative and simple expectation would be that an IL-12p40 deficiency may lead to the creation of Th2 environment due to the lack of Th1 inducing IL-12p70. Thus, it may lead to the more susceptible condition for the development of Th2-mediated diarrhea. Although we do not have any specific data to negate the latter possibility, one possible explanation would be that the deficiency of IL-12p70 formation in IL-12p40 KO mice lead to the lack of ability to active antigen presenting cells. IL-12p70 deficiency may

result in the absence of induction antigen-specific T cell response including the pathological Th2-type cells. It has been shown that IL-12 or IL-12-induced IFNγ can directly activate antigen presenting cells.[40] To address the issue, a series of interesting experiment would be the adaptive transfer of large intestinal MØ, DC, and epithelial cells into IL-12p40 and/or p35 KO mice. These experiments are, of course, planned for our future study.

Recently, it has been suggested that IL-12 is also one of the key cytokines for the regulation of the intestinal immune response.[41] Mouse IL-12p40 is produced as monomer and homodimer five to ninety times as frequently as IL-12p70 *in vivo* and *in vitro*,[40,42] implying the existence of additional immunological roles for IL-12p40. An interesting possibility would be that excess production of the monomeric and/or the homodimeric form of IL-12p40 could be a key contributing factor to the maintenance of immunological homeostasis at the mucosal compartment. Interestingly, our present findings demonstrate that over-expression of IL-12p40 occurred only in the large but not the small intestine following oral exposure to high doses of protein antigen. At the present time, we cannot offer any specific explanation for this distinct localization of IL-12p40. However, an interesting possibility would be that the expression of negative regulators for IL-12, including sCD40L and IL-10R,[14,43] could differ between the small and large intestine. To support this possibility, epithelial cells have been shown to express CD40 and IL-10R.[34,44] Since the large intestinal tract is continuously exposed to overloaded microflora, the level of co-stimulatory molecule expression such as CD40 by large intestinal epithelial cells could be lower to avoid unnecessary inflammatory responses. Thus, the large intestinal tract may form an immunological environment favoring the generation of IL-12p40. This interesting possibility is currently being tested in our laboratory.

In summary, our results demonstrated that locally produced IL-12p40 contribute to the Th2 cell generation of pathological polarization in the large intestine of OVA-induced allergic diarrhea. This study provides the first evidence for the association of over-expressed IL-12p40 from intestinal epithelial cells, DC and MØ, in the development of allergic diarrhea. Thus, the application of anti-IL-12p40 mAb resulted in the reduction of disease incidence and severity. Further, the disease development was completely eliminated in the deletion of IL-12p40 gene. Taken together, our studies provide an opportunity to consider that anti-IL-12p40 mAbs may be an alternative therapeutical regimen for the control of allergic intestinal disease.

Acknowledgments

We thank members of the Mucosal Immunology Group at Osaka University, The University of Tokyo and The University of Alabama at Birmingham, Immunobiology Vaccine Center for their helpful comments, Dr. Kimberly K. McGhee for editorial help, and Ms. Kelly Stinson and Ms. Sheila Turner for their help in the preparation of this manuscript.

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Intestinal villous M cells: An antigen entry site in the mucosal epithelium

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Communicated by Roy Curtiss, Washington University, St. Louis, MO, February 11, 2004 (received for review September 20, 2003)

M cells located in the follicle-associated epithelium of Peyer's patches (PP) are shown to be the principal sites for the sampling of gut luminal antigens. Thus, PP have long been considered the gatekeepers of the mucosal immune system. Here, we report a distinct gateway for the uptake of gut bacteria: clusters of non-follicle-associated epithelium-associated *Ulex europaeus* agglutinin (UEA)-1$^+$ cells, which we have designated intestinal villous M cells. Interestingly, villous M cells are developed in various PP [or gut-associated lymphoid tissue (GALT)]-null mice, such as *in utero* lymphotoxin β receptor (LTβR)-Ig-treated, lymphotoxin α (LTα)$^{-/-}$, tumor necrosis factor/LT$\alpha^{-/-}$, and inhibition of differentiation 2 (Id2)$^{-/-}$ mice. Intestinal villous M cells have been observed to take up GFP-expressing *Salmonella*, *Yersinia*, and *Escherichia coli*-expressing invasin, as well as gut bacterial antigen for subsequent induction of antigen-specific immune responses. Thus, the identified villous M cells could be an alternative and PP-independent gateway for the induction of antigen-specific immune responses by means of the mucosal compartment.

The huge intestinal surface area is physically protected by a layer of tightly joined epithelial cells, which prevent most enteric environmental antigens from penetrating the host (1). However, entry into the host is made possible by a special gateway, comprised of M cells, located over organized mucosal lymphoid follicles such as Peyer's patches (PP). The M cells, characterized by an irregular brush border and reduced glycocalyx, efficiently take up and transport a wide variety of macromolecules and microorganisms from the gut lumen to the inside of the PP (2–6), which contain all of the necessary lymphoid cells for the induction and regulation of antigen-specific IgA responses (7). However, the origin of M cells and the regulation of their development are not understood. A previous study (8) showed that i.v. injection of PP lymphocytes into severe combined immunodeficient mice resulted in formation of new lymphoid follicles and follicle-associated epithelium (FAE) with typical M cells. A similar phenomenon was seen by using *in vitro* studies in which coculture with B lymphocytes triggered the conversion of enterocyte cell lines into M cell-like cells (9). Further, B cells have recently been proposed to play a role in the organogenesis of the mucosal immune barrier (10). Two different B cell-null mice, lacking expression of either μ membrane exon or the J_H segment of Ig genes, showed drastic reduction of FAE size and M cell numbers (10). In contrast, a recent study (11) demonstrated that the absence of mature T and B cells does not prevent the formation of FAE and M cells, and signaling of lymphotoxin (LT) α/β from non-B and non-T cells plays a critical role in formation of M cells in FAE of PP.

The common mucosal immune system (CMIS), which connects the inductive (e.g., PP) and effector (e.g., lamina propria; LP) sites, has been shown to be a central pathway for the induction of antigen-specific IgA immune responses in the gastrointestinal tract (7). For example, oral administration of *Salmonella typhimurium* leads to the transport of the bacterial antigen from the lumen of the intestinal tract into the PP by means of M cells for the initial priming of antigen-specific CD4$^+$ T cells and IgA-committed B cells (12). These antigen-sensitized cells leave the PP and contribute to the subsequent induction of *Salmonella*-specific IgA response in the distant intestinal LP by means of CMIS. In addition to the well-characterized CMIS-dependent IgA induction pathway, recent evidence suggests the presence of an additional IgA induction pathway that is independently operated from the PP-originated CMIS (13–15). Interestingly, it also has been reported that induction of intestinal mucosal IgA against the commensal bacteria was independent from T cell help and organized lymphoid tissue (16). Further, our recent study (17) has demonstrated that antigen-specific IgA antibody responses can be induced in the absence of PP. These studies imply the existence of a PP-independent mucosal immune pathway for dietary antigen and bacteria uptake.

A recent study (18) has suggested that the invasion gene (SPI1)-deficient *S. typhimurium* can be disseminated from the intestinal epithelium to the systemic compartment in the absence of PP-associated M cells by means of the CD18-dependent pathway. Further, dendritic cells in the lamina propria of the small intestine expressing tight junction protein offer another possible antigen uptake site (19). Thus, intestinal DCs are capable of extending dendrites to the lumen side by opening the tight junction. However, the exact mechanism for inducing Ag-specific immune responses independently of PP requires further elucidation.

In this study, we have discovered intestinal villous M cells, which serve as an antigen gateway for the sampling of gut bacteria and subsequent induction of Ag-specific immune responses in a PP-independent manner. These lines of study are crucial for understanding the mechanisms of antigen uptake from the gut lumen, and for the rational design of effective mucosal vaccines and optimal drug delivery across the gut.

Experimental Procedures

Mice. BALB/c and C57BL/6 mice were purchased from CLEA Japan (Tokyo). LTβR-Ig fusion protein-treated and tumor necrosis factor (TNF) and LTα double knockout (TNF/LT$\alpha^{-/-}$; 129 × C57BL/6) mice were generated as described (20, 21). LT$\alpha^{-/-}$ mice (C57BL/6) were obtained from The Jackson Laboratory. Inhibi-

Abbreviations: PP, Peyer's patches; FAE, follicle-associated epithelium; LT, lymphotoxin; TNF, tumor necrosis factor; WGA, wheat germ agglutinin; UEA, *Ulex europaeus* agglutinin; TRITC, tetramethylrhodamine B isothiocyanate; IEC, intestinal epithelial cell; ILF, isolated lymphoid follicle; GALT, gut-associated lymphoid tissue; Id2, inhibition of differentiation 2.

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www.pnas.org/cgi/doi/10.1073/pnas.0400969101

tion of differentiation 2 $(Id2)^{-/-}$ mice (129/Sv) were generated as described (22).

M Cell Staining. A standard lectin staining procedure was used for the detection of murine M cells (23). Mucus-free small intestine of naive BALB/c or C57BL/6 mice, with or without PP, was fixed in 4% paraformaldehyde for 1 h, washed, and then blocked with 10% FBS in PBS containing 0.1% glycine. A lectin-labeling experiment was performed with *Ulex europaeus* agglutinin (UEA) conjugated with tetramethylrhodamine B isothiocyanate (TRITC) (UEA-1-TRITC, Vector Laboratories) and wheat germ agglutinin (WGA) conjugated with FITC (WGA-FITC) at a concentration of 20 μg/ml for 2 hr. After being rinsed in PBS, samples were stored in a Tris-buffered solution containing 30% glycerol and 0.1% NaN_3. The specimens were examined in a Bio-Rad MRC-600 confocal imaging system (Bio-Rad). Alkaline phosphatase activity and alcian blue staining were assessed on whole fixed small intestine as described (11). In addition, scanning and transmission electron microscopy analyses were performed for the characterization of M cells (see *Supporting Experimental Procedures*, which is published as supporting information on the PNAS web site).

Antigen Uptake *in Situ*. *S. typhimurium* PhoPc strain transformed with the pKKGFP plasmid was kindly provided by F. Niedergang (24, 25). Further, GFP-expressing *Yersinia pseudotuberculosis*, *Escherichia coli*-invasin, and *E. coli* were prepared by the method described (26, 27). Mice were anesthetized by i.p. injection of 2 mg of ketamine (Sigma) per mouse. Segments ≈10 cm long of the small intestine of TNF/LT$\alpha^{-/-}$ mice and wild-type mice were ligated at both ends with surgical thread. GFP-expressing bacteria (5×10^8) were suspended in 1.0 ml and inoculated into the loop and incubated *in situ*. Ten minutes later, PP and the intestinal segments (without PP) were removed and extensively washed with cold PBS and RPMI medium 1640 including gentamycin (100 μg/ml). Intestinal epithelial cells (IECs) were isolated from PP and the intestinal segments as described (28), then fixed in 4% paraformaldehyde, washed with 10% FBS in PBS, and labeled with UEA-1-TRITC. The percentage of double-positive IECs was analyzed on a FACSCalibur flow cytometer (Becton Dickinson). In selected mice, whole-mounted small intestinal segments were processed for confocal microscopy as described above. To remove weakly adhered and/or extracellular bacteria, vigorous washing with cold PBS and RPMI medium 1640 containing gentamycin were adopted during the process of isolation of villous epithelium including M cells and epithelial cells after infection with bacteria. Gentamycin was selected as the antibiotic due to its lethal effects on *Salmonella* (29). Therefore, our present data include only *Salmonella* that had strongly adhered and was intracellular but not *Salmonella* that was weakly adhered and extracellular.

Immunization. The recombinant *S. typhimurium* BRD 847 strain used in the immunization study is a double *aroA aroD* mutant that expresses the nontoxic, immunogenic 50-kDa ToxC fragment of tetanus toxin from plasmid pTET*nir*15 under the control of the anaerobically inducible *nirB* promoter (r*Salmonella*-ToxC) (30). For the control, r*Salmonella* that are not expressing ToxC were adopted. Recombinant *Salmonella* organisms were resuspended in PBS to a concentration of 2.5×10^{10} bacteria per ml. Bacterial suspensions were orally administered by gavage (0.2 ml per mouse). Ab titers in serum were determined by ELISA, as described elsewhere (17).

Data Analysis. Data were expressed as mean ± SD and evaluated by the Mann–Whitney *U* test. *P* values of <0.05 were assumed to be statistically significant.

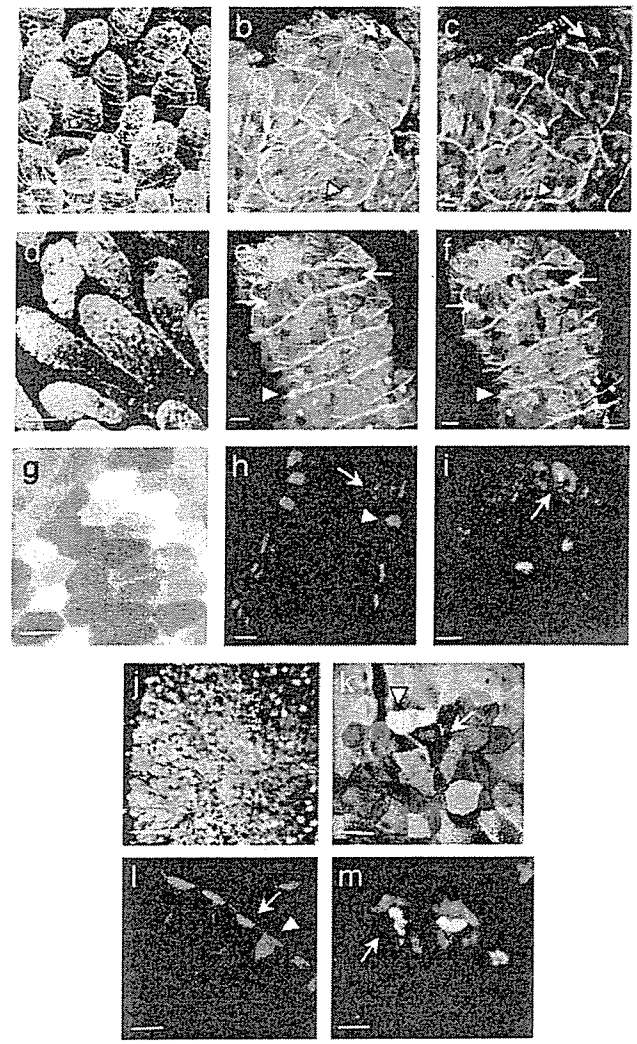

Fig. 1. Confocal view of UEA-1$^+$ cells in villous epithelium (*a–i*) and FAE of PP (*j–m*) isolated from naive BALB/c mice. M cell- and columnar epithelial cell-specific UEA-1-TRITC and WGA-FITC, respectively, were applied to the whole-mount preparation of the small intestine (*a–f*, *j*, and *k*). M cells were stained by UEA-1 (red, arrow), enterocytes by WGA (green), and goblet cells by UEA-1 and WGA (yellow, arrowhead). Villous M cells were found as two different distribution forms, dense (*a* and *b*) and diffuse (*d* and *e*) types. In contrast to the epithelial and goblet cells, M cells in the villous epithelium were completely negative to the WGA staining (*c* and *f*). Frozen sections were prepared and stained with UEA-1-TRITC alone (*h* and *l*) or with UEA-1-TRITC and B220 mAb-FITC (*i* and *m*) and the M cells were shown to have a pocket membrane and pocket lymphocytes (arrow) whereas the goblet cells do not (arrowhead). M cells were doubly negative cells for alkaline phosphatase activity demonstrated by red/pink color substrate, and alcian blue staining (white; *g*). The scale bar for *a*, *d*, and *j* is 50 μm; for *b*, *c*, *e*, *f*, *h*, and *l* is 20 μm; and for *g*, *i*, *k*, and *m* is 10 μm.

Results

Identification of Clusters of UEA-1$^+$ Cells in the Intestinal Villous Epithelium. M cells have been thought to be associated with, and to develop only in, the dome epithelium (or FAE) of mucosa-associated lymphoid tissues, e.g., PP. However, using confocal image analysis of whole-mount murine intestine stained with TRITC-conjugated UEA-1 and FITC-labeled WGA, we have found UEA-1$^+$WGA$^-$ cells not only in the FAE region of PP (Fig. 1 *j–l*), but also in the villous epithelium (Fig. 1 *a–i*). UEA-1, which possesses specificity for carbohydrate structures containing α(1–2)-fucose, selectively binds to the entire plasma membrane of PP M

cells but not to WGA^+ columnar epithelial cells (23). To further confirm the specificity of UEA-1 staining, we have performed a blocking experiment using 50 mM soluble fucose. Preincubation of the UEA-1 with soluble fucose for 1 hr clearly blocked UEA-1 staining in fluorescence-activated cell sorter (FACS, Becton Dickinson) and immunohistochemistry analyses further indicating the specificity of the UEA-1 staining method (data not shown). Interestingly, two forms of villous $UEA\text{-}1^+WGA^-$ cells, i.e., dense and diffuse, may be distinguished on the basis of the density of $UEA\text{-}1^+WGA^-$ cells (Fig. 1 *a* and *b* vs. *d* and *e*).

Our study revealed that these newly identified villous $UEA\text{-}1^+WGA^-$ cells share features with PP M cells but differ from goblet and columnar epithelial cells. Analysis of frozen sections of intestinal villi stained with TRITC-UEA-1 reveals that the villous $UEA\text{-}1^+$ cells possess the characteristic feature of M cells in PP FAE, i.e., a unique subdomain of the basolateral membrane, also known as the pocket membrane (Fig. 1*h*). The pocket lymphocytes were further confirmed by the staining with TRITC-UEA-1 and FITC-B220 mAb in villous $UEA\text{-}1^+$ cells (Fig. 1*i*) as well as in PP M cells (Fig. 1*m*).

Although they possess some affinity for UEA-1, goblet cells, unlike M cells, are capable as well of binding to WGA, making them doubly positive cells (UEA^+ WGA^+; Fig. 1 *b*, *e*, and *k*). In contrast to the epithelial and goblet cells, $UEA\text{-}1^+$ cells in the villous epithelium were completely negative for WGA staining (Fig. 1 *c* and *f*). Further, goblet cells are morphologically distinguished from M cells in that they do not possess the characteristic pocket membrane (Fig. 1 *h* and *i*). Intestinal columnar epithelial cells have high alkaline phosphatase (ALP) activity demonstrating with red or pink color, as do goblet cells stained with alcian blue, but M cells have neither of these features (11). Like PP M cells, villous $UEA\text{-}1^+$ cells in whole-mount intestinal samples were found to be negative for ALP activity and alcian blue staining (Fig. 1*g*). Thus, the $UEA\text{-}1^+$ cells, shown by our study to be analogous to PP M cells, have been designated villous M cells.

Development of Clusters of Villous $UEA\text{-}1^+$ M Cells in the Various PP-Null Mice. To further support this view, we examined whether villous M cells can develop in PP [or gut-associated lymphoid tissue (GALT)]-deficient mice, such as *in utero* LTβR-Ig-treated (21), $LT\alpha^{-/-}$ (31), $TNF/LT\alpha^{-/-}$ (20), and $Id2^{-/-}$ mice (22). We found M cells with the characteristic $UEA\text{-}1^+$ WGA^- staining in the tip regions of intestinal villi of all PP-deficient mice (Fig. 2), thus documenting the presence of an FAE-independent M cell developmental pathway. This view was further supported by the presence of M cells in $TNF/LT\alpha^{-/-}$ mice lacking newly described isolated lymphoid follicle (ILF) in addition to PP (data not shown). To define the distribution and number of villous M cell population in wild-type mice and GALT-null mice, we determined the frequency of the dense type of villous M cells in whole small intestine using the confocal imaging system. Approximately 40–50 dense-type villous M cell clusters were found per whole small intestine of wild-type mice. Similarly, ≈50–60 villous M cell clusters were found in the whole small intestine of $TNF/LT\alpha^{-/-}$ mice, one of the representative GALT-null mice. The finding of similar numbers of villous M cells in the GALT-null and wild-type mice could suggest that the development of villous M cells is completely independent of GALT and FAE.

Attachment and Internalization of Bacteria by Villous M Cells. A further experiment was performed to gauge the ability of villous M cells to take up pathogenic microorganisms. Ligated small intestinal loops from wild-type mice were inoculated with r*Salmonella typhimurium* expressing green fluorescence (r*Salmonella*-GFP), *Yersinia pseudotuberculosis* (*Yersinia*-GFP), *E. coli*-expressing *Yersinia* invasin (*E. coli*-invasin-GFP), and wild-type *E. coli*-GFP. After a 10-min incubation with each bacteria *in situ*, sequential immunohistologic analyses of ligated small intestinal loops directly demonstrated the

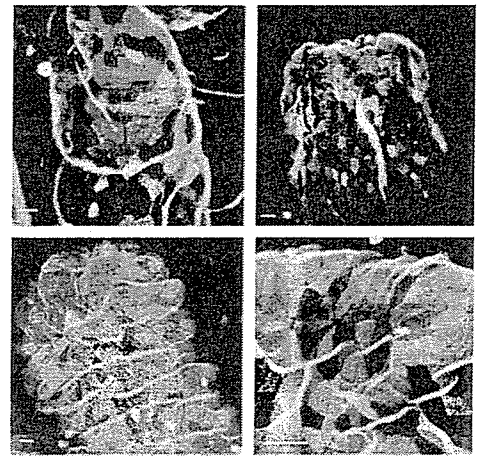

Fig. 2. The presence of villous M cells in PP-null mice, such as *in utero* LTβR-Ig-treated C57BL/6 mice (*Upper Left*), $LT\alpha^{-/-}$ mice of C57BL/6 background (*Upper Right*), $TNF/LT\alpha^{-/-}$ mice of 129 × C57BL/6 background (*Lower Left*), and $Id2^{-/-}$ mice of 129×Sv background (*Lower Right*). The scale bar for all pictures is 10 μm. The whole-mount preparations of small intestine were stained with FITC-WGA and TRITC-UEA-1.

presence of r*Salmonella*-GFP in $UEA\text{-}1^+$ cells in the villous epithelium of wild-type mice (Fig. 3 *Aa* and *Ab*) and $TNF/LT\alpha^{-/-}$ mice (Fig. 3 *Ae* and *Af*). In addition, *Yersinia*-GFP was also specifically adhered to villous $UEA\text{-}1^+$ cells of wild-type mice (Fig. 3 *Ac* and *Ad*) and $TNF/LT\alpha^{-/-}$ mice (Fig. 3 *Ag* and *Ah*). Immunohistologic analyses using frozen sections showed that r*Salmonella*-GFP was located in the apical membrane regions of villous $UEA\text{-}1^+$ cells (Fig. 3 *Ai* and *Aj*). To show the ability of villous $UEA\text{-}1^+$ cells to take up bacteria, we performed an ileal loop infection experiment using *rSalmonella*-GFP and analyzed the localization of bacteria with sequential confocal microscopy (Fig. 3*B*). Sequential Z plans of whole mount staining revealed the localization of *rSalmonella*-GFP in the intracellular region (Fig. 3 *B a–c*). In addition, *rSalmonella*-GFP was found in the intracellular region of villous $UEA\text{-}1^+$ cells prepared by cytospin (Fig. 3*Bd*).

Intestinal epithelial cells were further isolated from villous epithelium and PP after ileal loop injection of the microorganism expressing GFP, and then counterstained with TRITC-UEA-1 for flow cytometry analysis. We found a higher frequency of r*Salmonella*-GFP-, *Yersinia*-GFP-, or *E. coli*-invasin-GFP-containing cells in the fraction of $UEA\text{-}1^+$ cells than in the $UEA\text{-}1^-$ cells isolated from villous epithelium (Fig. 3*C*), and similar patterns were noted for the $UEA\text{-}1^+$ and $UEA\text{-}1^-$ cells isolated from the dome region of PP. In addition, high numbers of r*Salmonella*-GFP-, *Yersinia*-GFP-, or *E. coli*-invasin-GFP-containing cells were also recovered from $UEA\text{-}1^+$ but not $UEA\text{-}1^-$ cells isolated from the villous epithelium of $TNF/LT\alpha^{-/-}$ mice lacking GALT (Fig. 3*C*). Taken together, these results indicate that villous M cells have the ability to take up several different bacteria from the lumen known to be taken up by FAE-M cells.

Scanning and Transmission Electron Microscope Analysis of Villous M Cells. Scanning electron microscopy (SEM) of the villous M cells revealed a hallmark feature of M cells, i.e., a depressed surface with short and irregular microvilli (Fig. 4 *a*, *b*, and *c*), seen also in the M cells of PP (Fig. 4 *d* and *e*). Transmission electron microscopy analysis also showed the binding of gold particle-conjugated $UEA\text{-}1^+$ cells in the villous M cells (Fig. 4 *f* and *g*). Further, the presence of infiltrating mononuclear cells was also seen in the pocket of villous M cells (Fig. 4*h*). The SEM also demonstrated the binding of bacteria to the membrane of villous M cells in the small intestine of FAE-null $TNF/LT\alpha^{-/-}$ mice after intestinal exposure

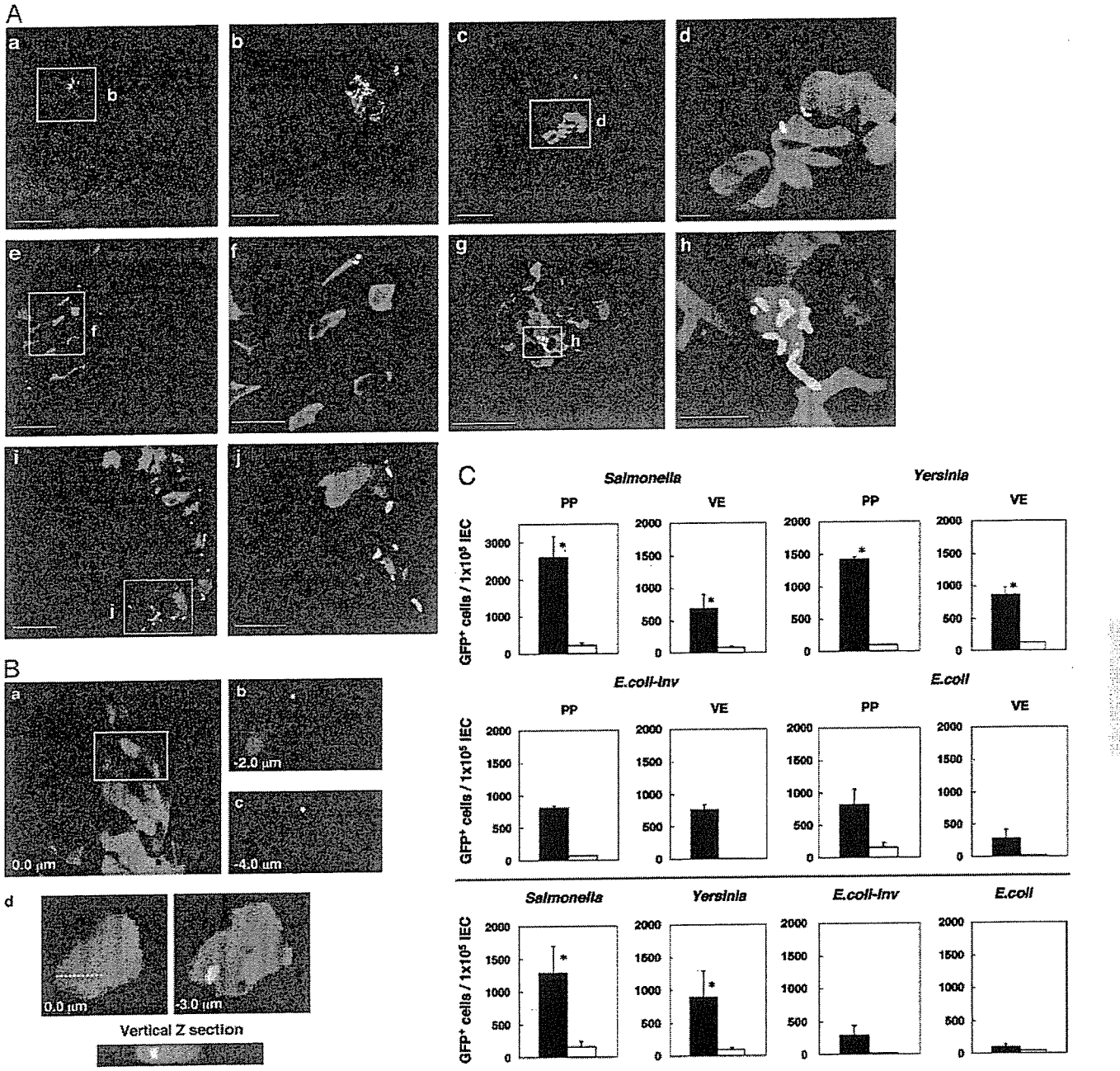

Fig. 3. (*A*) Immunohistochemistry for antigen uptake by UEA-1$^+$ villous M cells. Each panel shows histological features for sampling of GFP-expressing *Salmonella* (*a*, *b* and *e*, *f*) and *Yersinia* (*c*, *d* and *g*, *h*) by UEA$^+$ cells in the small intestine of wild-type (*a–d*) and PP-null TNF/LT$\alpha^{-/-}$ mice (*e–j*). Whole mount (*a–d* and *e–h*) and frozen sections of small intestine after exposure of GFP-expressing *Salmonella* were prepared and stained with UEA-1-TRITC (*i* and *j*). The scale bars are as follows: for *a*, *c*, and *g*, 50 μm; for *b*, *e*, and *i*, 20 μm; and for *d*, *f*, *h*, and *j*, 10 μm. (*B*) Localization of GFP-expressing *Salmonella* in the intracellular region of UEA-1$^+$ villous M cells. An ileal loop infection experiment using *rSalmonella*-GFP was performed for 30 min, and whole-mount tissues and UEA-1$^+$ IEC cells were analyzed by sequential confocal planar microscopy. Sequential Z plans of whole-mount staining revealed the localization of *rSalmonella*-GFP in the intracellular region of villous UEA-1$^+$ cells (*a*, *b*, and *c*). Further, the cytospin analysis revealed that *rSalmonella*-GFP also existed in the intracellular region of villous UEA-1$^+$ cells (*d*). (*C*) Antigen uptake by UEA-1$^+$ villous M cells. Cells (5×10^8) of GFP-expressing *S. typhimurium* PhoPc (*Salmonella*), *Y. pseudotuberculosis* (*Yersinia*), *E. coli*-invasin (*E. coli*-Inv), and *E.-coli* were administered into a 10-cm loop of the small intestine of naive wild-type mice (*Top* and *Middle*) or TNF/LT$\alpha^{-/-}$ mice (*Bottom*). After 10 min of incubation *in situ*, IECs were isolated from PP and villous epithelium. After being fixed with 4% paraformaldehyde, IECs were stained by UEA-1-TRITC, and uptake efficiency was analyzed by fluorescence-activated cell sorter (FACS). Data demonstrate the frequency of GFP$^+$ cells in the UEA$^+$ (filled bar) and UEA$^-$ (open bar) cells isolated from PP and villous epithelium (VE). The results represent the mean values ± SD from three separate experiments (three mice per group). *, $P < 0.05$ vs. the UEA$^-$ IEC group.

of *Salmonella* (Fig. 4 *i–l*). These findings provide supportive evidence that the newly identified villous M cell formed an alternative gateway for antigen sampling and/or entry from the lumen of intestinal villous epithelium. These findings provide evidence that M cells are developed and localized in the villous epithelium as well as in the FAE of PP.

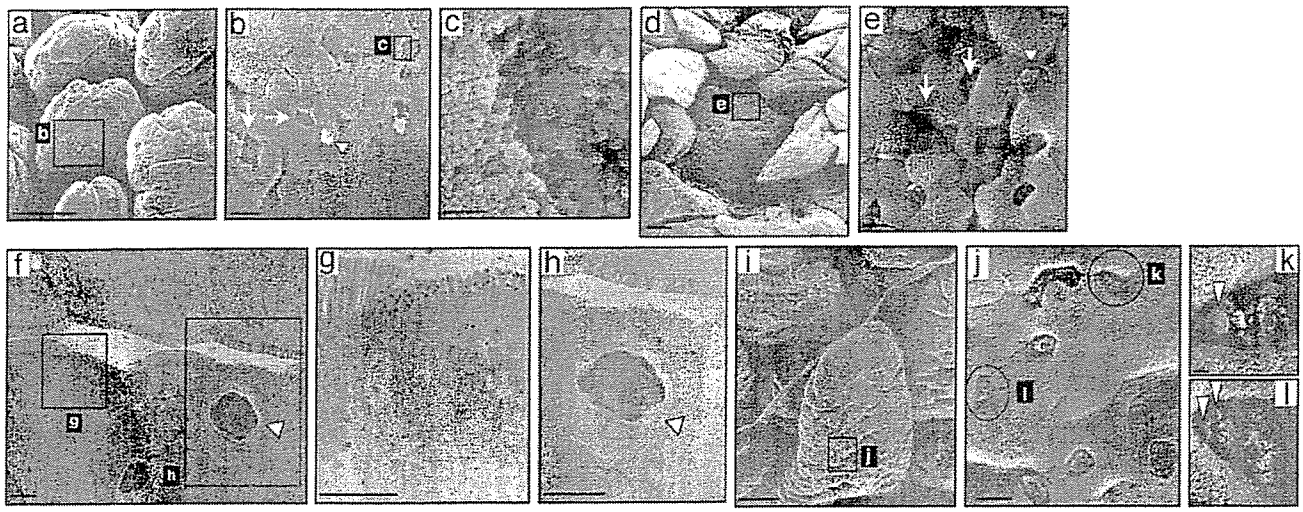

Fig. 4. Scanning and transmission electron microscopy of M cells in villous epithelium and FAE. Scanning electronic microscopy demonstrates that the M cells (arrow) in villous epithelium (*a–c*) and PP (*d* and *e*) are distinguished from enterocytes and goblet cells (arrowhead) by their relatively depressed and dark brush border. A transmission electron microscopy view of villous M cells shows short stub-like microvilli (*f*, *g*, and *h*) and the presence of infiltrating mononuclear cells in the pocket of villous M-cells (*h*; arrowhead). (*i–l*) The presence of villous M cells and the uptake of bacteria in the villous epithelium (arrowhead) after intestinal exposure of *Salmonella* (see Fig. 3 legend) in PP-deficient TNF/LT$\alpha^{-/-}$ mice. The scale bars are as follows: for *a*, *d*, and *i*, 50 μm; for *b*, *e*, *f*, and *j*, 5 μm; for *g* and *h*, 1.0 μm; and for *c*, *k*, and *l*, 0.5 μm.

Induction of Ag-Specific Immune Responses in PP-Deficient Mice. Our next experiments sought to examine whether antigen-specific antibody responses could be induced in GALT-deficient mice by means of the villous M cells. When GALT-null mice with TNF/LTα gene deficiency and wild-type mice were immunized orally with r*S. typhimurium* BRD 847 expressing a 50-kDa ToxC fragment of tetanus toxin (r*Salmonella*-ToxC), titers of tetanus toxoid (TT)-specific serum IgG antibodies were as high in the serum of the TNF/LT$\alpha^{-/-}$ mice as in orally immunized wild-type mice (Fig. 5). Expectedly, levels of TT-specific serum IgG antibody titers were not detectable when wild-type and TNF/LT$\alpha^{-/-}$ mice were orally immunized with r*Salmonella* not expressing ToxC (under the 5 of reciprocal $\log_2$ titer in Fig. 5). These findings suggest that the villous M cells are an important antigen-sampling site for the induction of antigen-specific immune responses to gastrointestinal environmental antigens.

Discussion

Because there is currently no reliable identified gene and corresponding antigen marker that positively identified M cells, the phenotype of M cells is defined by a combination of criteria including (3, 4) (*i*) the presence of the fucose epitope defined by the lectin UEA on M cell membrane, (*ii*) short and irregular microvilli, (*iii*) endocytic activity and ability to take up bacteria as well as macromolecules, and (*iv*) an intraepithelial pocket that allows a cluster of lymphocytes to be located in the epithelium. Based on our present results, villous M cells share all of the identifying features necessary to identify M cells found in the FAE of PP. Although M cell development has been thought to depend on FAE in organized mucosal lymphoid tissues, our results provide evidence that it can occur in the villous epithelium even in the absence of FAE. Further, these villous M cells are a gateway for entry or sampling of bacteria (e.g., *Salmonella*-, *Yersinia*-, and *E.-coli*-expressing invasin) for the subsequent induction of antigen-specific immune responses.

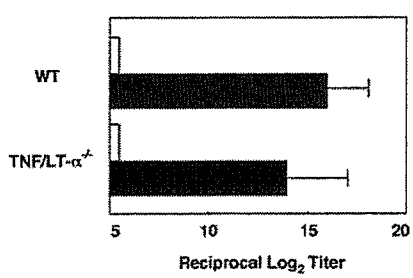

Fig. 5. Induction of Ag-specific immune responses in PP-deficient mice. Shown are PP-deficient (TNF/LT$\alpha^{-/-}$) and wild-type mice, which were orally immunized with r*Salmonella*-ToxC (filled bar) or r*Salmonella* alone (open bar). Serum samples were obtained 21 days after oral immunization for the assessment of tetanus toxoid (TT)-specific antibody responses by ELISA. The results represent the mean values ± SD from three separate experiments (three mice per group). There is no statistically significant difference between TNF/LT$\alpha^{-/-}$ and wild-type mice analyzed by unpaired Mann–Whitney *U* test.

M cells have been identified and documented only in the FAE-associated epithelium and occasionally on villi immediately adjacent to the lymphoid follicle (4, 32). A previous study (32) indicated that isolated M cells were found in the villous epithelium near the PP of the rabbit small intestine. In addition, clusters of UEA-1$^+$ cells in the small intestinal villi of conventional mice have been reported (33). These two studies suggested the existence of UEA-1$^+$ cells in the small intestinal villi of rabbit and mouse but did not address their identity or biological function. In this regard, our present study provides evidence of the existence of M cells in the villous epithelium away from PP of not only wild-type mice but also GALT-null mice. Further, our present results directly demonstrate the functional aspect of the villous M cells as a gateway for bacteria. Thus, we have substantially advanced the case that villous M cells are distinct from FAE-associated M cells in PP and have further shown that these villous M cells are a biologically important component of the mucosal immune system.

As discussed above, M cells reportedly are occasionally found in the villous epithelium adjacent to rabbit PP (32); however, we stress that villous M cells are located quite a distance from PP. Although the exact source of M cells has yet to be pinpointed, it is widely held that their development and localization are always associated with the organized lymphoid tissue of mucosal surfaces (e.g., PP). Our findings presented here, however, challenge this common assumption by providing evidence that M cells can be developed in villous epithelium in the absence of the FAE thought to be necessary to

their development in the organized mucosal lymphoid tissue. The diverse cellular phenotypes in the intestinal epithelium arise from crypt stem cells whose differentiation pathways can be modified by endogenous and exogenous influences (33–37). A previous study (34) demonstrated that *Streptococcus pneumoniae*-treated FAE tissues showed a marked increase in both IEL and epithelial cells with morphological and functional features of M cells. Further, enterocytes located in the peripheral of the FAE were converted into operational M cells as early as one hour after *in vivo* exposure to *S. pneumoniae* (32). Interestingly, expression of α1,2-linked fucosylated glyconjugates in the ileal epithelium was induced by the flora (33). Further, our unpublished data indicate that significantly increased numbers of UEA-1$^+$ cells in the villous epithelium of both wild-type and PP-null mice were detected after *in vivo* exposure to *S. typhimurium*. Therefore, it is possible that newly identified villous M cells can be developed from epithelial cells in response to foreign antigens and/or pathogens in the gut lumen. An interesting possibility would be that these UEA-1$^+$ crypt cells could be programmed to develop into the villous UEA-1$^+$ M cells after exposure to the exogenous microorganisms.

M cells in the FAE provide an entry site for pathogens, such as *S. typhimurium*, *Mycobacterium bovis*, *Shigella flexneri*, *Y. enterocolitica* and retroviruses (4, 38–40). It is well known that the invasion genes of the *Salmonella* pathogenicity island (SPI1) are necessary for the entry of *S. typhimurium* into FAE-M cells and epithelial cells (38, 41). However, SPI1-deficient *Salmonella* is transported from the gastrointestinal tract to the blood stream by CD18-expressing phagocytes, and CD18-deficient mice were shown to be resistant to orally administered *Salmonella* (18). Overall, it seems likely that several cell types, including M cells, epithelial cells, and CD18-expressing macrophages, are involved in permitting the penetration of *Salmonella*. On the other hand, previous studies have showed that *Y. enterocolitis* selectively and specifically invades the FAE of PP by means of M cells but not by means of other cells (42, 43). Interestingly, it has been suggested $\alpha_4\beta_1$ integrin is expressed on the apical membranes of M cells but not on villous or dome epithelial enterocytes, implying that this integrin may be exploited by *Yersinia* to attach to and invade the M cells (44). Further, invasin mediates uptake of *Y. pseudotuberculosis* into mammalian cells through binding with β1-chain integrins with high affinity (45, 46). In light of these complexities, the fact that villous M cells and FAE-associated M cells in PP sampled GFP-expressing *Salmonella*, *Yersinia*, and *E. coli*-invasin suggests that villous M cells likely possess a capacity of playing as professional bacteria translocating cells.

A recent study (13) has provided new evidence that IgA-specific B cell responses including isotype-switching can be induced in intestinal lamina propria without the influence of PP. In addition, our recent study showed that ILF in the small intestine are structurally and functionally similar to the PP and contain M cells on their FAE region (47). To eliminate the possible role of M cells associated with the ILF for antigen sampling, we used TNF/LT$\alpha^{-/-}$ mice, which lack both PP (17) and ILF (unpublished data). Interestingly, high numbers of GFP-expressing *Salmonella*, *Yersinia*, and *E. coli*-invasin were recovered from UEA-1$^+$ but not UEA-1$^-$ cells isolated from the villous epithelium of TNF/LT$\alpha^{-/-}$ mice although the total uptake of GFP$^+$ eukaryotic cells was less pronounced than in wild-type mice (Fig. 3*C*). Together with the data for the induction of antigen-specific antibody responses after oral immunization in these PP- and ILF-deficient mice (Fig. 5), our results provide a strong case that villous M cells are an alternative gateway of antigen entry for the mucosal immune system. On the other hand, a recent study (19) showed that antigen sampling occurs across the non-FAE by mucosal intraepithelial dendritic cells. Thus, it is still possible that antigen-specific immune responses seen in TNF/LT$\alpha^{-/-}$ mice could be initiated by means of these intraepithelial dendritic cells.

In summary, our observations indicate that typical M cells can develop without the influence of the FAE associated with mucosal lymphoid tissue such as PP and in fact are present in villous epithelium. Moreover, villous M cells may be an alternative gateway for the penetration of pathogenic microorganisms as well as an additional antigen-sampling site for the induction of antigen-specific immune responses by means of the mucosal tissues.

We thank Mr. Takashi Wada for helpful technical assistance with scanning electron microscopy. We thank Drs. William R. Brown and Kimberly McGhee for editing the manuscript and Drs. Ichiro Takahashi, Jean-Pierre Kraehenbuhl, and Satoshi Fukuyama for helpful discussions and suggestions. This work was supported by grants from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation (JST), the Ministry of Education, Science, Sports, and Culture, and the Ministry of Health and Welfare in Japan, as well as by SRC fund to IRC at the University of Ulsan from Korea Science and Engineering Foundation (KOSEF) and the Korean Ministry of Science and Technology.

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IgA Class Switch Occurs in the Organized Nasopharynx- and Gut-Associated Lymphoid Tissue, but Not in the Diffuse Lamina Propria of Airways and Gut[1]

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Secretory IgA plays a crucial role in the host immune response as a first line of defense. A recent demonstration of in situ IgA class switching in intestinal lamina propria provided an opportunity to reconsider the model for the homing of IgA-committed B cells characterized by distinctive trafficking patterns to effector sites. Those effector sites depend on the organized mucosa-associated lymphoid tissues as their site of induction. In this report we show the preferential presence of IgM^+B220^+ and IgA^+B220^+ cells belonging to pre- and post-IgA isotype class-switched cells in the organized mucosa-associated lymphoid tissues, such as nasopharynx-associated lymphoid tissues, isolated lymphoid follicles, and Peyer's patches, and the defect of those populations in the diffuse effector tissues, such as the nasal passage and intestinal lamina propria. Consistent with these findings, the expressions of a series of IgA isotype class switch recombination-related molecules, including activation-induced cytidine deaminase, $I\alpha$-$C\mu$ circle transcripts, and $I\alpha$-$C\mu$ circle transcripts, were selectively detected in these organized mucosa-associated lymphoid structures, but not in the diffuse mucosal effector sites. Taken together, these findings suggest that IgA isotype class switching occurs only in the organized mucosa-associated lymphoid organs (e.g., nasopharynx-associated lymphoid tissues, isolated lymphoid follicles, and Peyer's patches), but not in the diffuse effector tissues of the upper respiratory and gastrointestinal tracts. ***The Journal of Immunology,*** **2004, 172: 6259–6264.**

The mucosal immune system provides the first line of defense against the ingress of microbial pathogens during the physiological processes of inhalation and ingestion. One of the major components of the mucosal immune system is secretory IgA (S-IgA),[3] which is produced by the mucosal interaction of epithelial cells, IgA-committed B cells, and Th cells in the respiratory and gastrointestinal tracts (1, 2). Thus, mucosal effector sites such as the lamina propria of the upper respiratory and intestinal tract contain high numbers of IgA blast and plasma cells derived from postswitched IgA^+ B cells.

IgA-committed B cells that have undergone μ to α isotype class switching in nasopharynx-associated lymphoid tissue (NALT), Peyer's patches (PPs), and other mucosal inductive lymphoid organs are generally believed to migrate to diffuse mucosal effector tissues, including the nasal passage (NP) and intestinal lamina propria (i-LP) (3, 4). PPs show a high frequency of IgM^+ $B220^+$ B cells, which are a prerequisite for isotype class switching to IgA^+B220^+ B cells after antigenic or mitogenic stimulation in the presence of the isotype switch-inducing cytokine TGF-β (1, 5–8). PPs are an example of organized gut-associated lymphoid tissue (GALT) with germinal centers (GCs) and are thus considered to be a major site for the μ to α class switch recombination (CSR) involving activation-induced cytidine deaminase (AID), $I\alpha$-$C\mu$ circle transcripts (αCTs), and $I\mu$-$C\alpha$ transcripts (9, 10). Recent results, generated from analysis of AID-deficient mice, however, have suggested another pathway for the development of intestinal IgA plasma cells (11). IgA class switching was shown to occur in i-LP without the involvement of PPs or other organized mucosa-associated lymphoid tissues (MALT) containing GCs. In this study, IgM^+ $B220^+$ B cells in i-LP switched to IgA^+ B cells under the influence of TGF-β derived from i-LP stromal cells (11). These findings suggest that i-LP could have the immunological function of both effector and inductive sites. However, the concerns with that study were that the researchers did not have control of the compartment from which the B cells were harvested, either the diffuse lamina propria or isolated lymphoid follicles (ILFs) embedded in the lamina propria, due to the nature of AID-deficient mice. Because $AID^{-/-}$ mice exhibited enormous hyperplasia of ILFs, there was little of the diffuse lamina propria region left (11).

We recently identified ILFs as part of the organized GALT in mouse small intestine (12). ILFs are comprised of a single B cell

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Received for publication October 9, 2003. Accepted for publication March 11, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants-in-aid from Core Research for Engineering, Science, and Technology of Japan Science and Technology Corp.; the Ministry of Education, Science, Sports, and Culture of Japan; the Ministry of Health, Labor, and Welfare of Japan; and the Japanese Human Science Foundation.

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[3] Abbreviations used in this paper: S-IgA, secretory IgA; AID, activation-induced cytidine deaminase; CSR, class switch recombination; CT, cholera toxin; αCT, $I\alpha$-$C\mu$ circle transcript; GALT, gut-associated lymphoid tissue; GC, germinal center; ILF, isolated lymphoid follicle; i-LP, intestinal lamina propria; MC, mononuclear cell; MLN, mesenteric lymph node; NALT, nasopharynx-associated lymphoid tissue; NP, nasal passage; PP, Peyer's patch.

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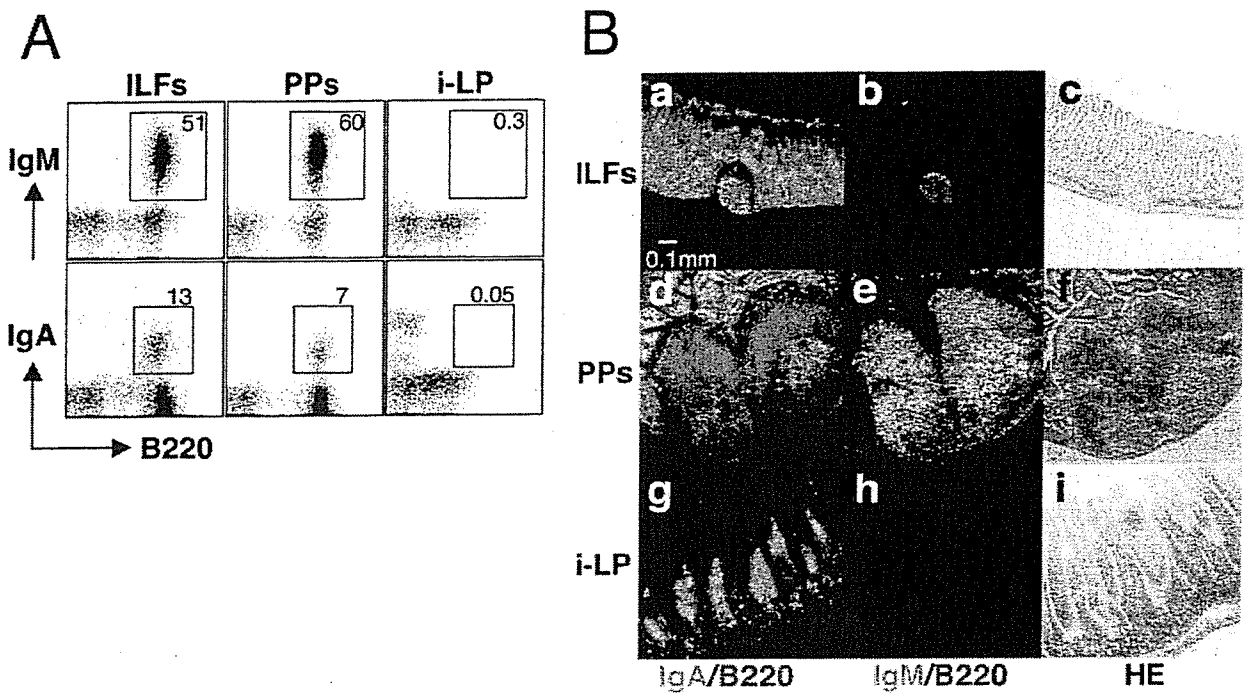

FIGURE 1. The accumulation of IgM^+B220^+ and IgA^+B220^+ cells in ILFs and PPs and their absence in the i-LP of mouse small intestine. *A*, MCs isolated from ILFs, PPs, and i-LP were stained with a panel of mAbs specific for IgM, IgA, and B220. The i-LP MCs were prepared after complete removal of ILFs and PPs. *B*, Consecutive tissue sections were stained with FITC-conjugated anti-IgA (*a*, *d*, and *g*), anti-IgM (*b*, *e*, and *h*), and PE-conjugated anti-B220 (*a*, *b*, *d*, *e*, *g*, and *h*). These tissues were also stained with HE (*c*, *f*, and *i*). Although cells expressing IgA or IgM (green) as well as those expressing B220 (red) were found in ILFs and PPs (*a*, *b*, *d*, and *e*), double-positive cells were hardly detected in i-LP (*g* and *h*), where IgA^+B220^- blast/plasma cells (green) were abundant (*g*). These sections were analyzed by confocal microscopy using a ×25 magnification. Results are representative of three separate experiments.

follicle with GCs overlaid by a follicle-associated epithelium containing Ag up-taking M cells that are similar to the follicle-associated epithelium of PPs. The presence of ILF tissue could provide additional mucosal inductive sites for the generation of IgA-committed B cells in the gastrointestinal tract (13). In fact, looking at the data overall, it is not possible to exclude the possibility that ordinary preparations of i-LP samples may contain IgM^+ $B220^+$ B cell fractions undergoing in situ class switching inside the GCs of these newly discovered murine ILFs (12).

For the upper respiratory tract, NALT has been shown to contain all the necessary molecular and cellular environments for the initiation of IgA B cell responses (2, 14, 15). NALT has also been identified as a region in which both IgA-committed B cells and memory-type IgA^+ B cells are generated (15). Nasal immunization has been shown to effectively induce Ag-specific IgA Ab responses via NALT for the upper respiratory tract, including the NP (2, 16). Thus, in the respiratory tract, NALT, with its inductive sites, and NP, with its effector sites, are considered to be part of the IgA-committed B cell-homing pathway analogous with PPs and i-LP in the gastrointestinal tract.

In this study, using the molecular and cellular analyses of transcription as well as immunocytochemistry and immunohistochemistry, NALT and GALT, including ILFs and PPs as well as NP and i-LP, were examined as examples of organized inductive and diffuse effector tissues, respectively. In this paper we report evidence that organized MALT, but not the diffuse lamina propria of the respiratory and intestinal tracts, play an essential role in the generation of IgA-committed B cells, especially in the μ to α isotype class switching for the mucosal immune system.

Materials and Methods

Mice

BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and used at 6–12 wk of age. In some experiments mice were nasally immunized with

Table I. *Distribution of different B cell subsets in organized mucosa inductive vs diffuse effector tissues*[a]

B Cell Subsets		Respiratory Tract		Intestinal Tract		
				Inductive		
sIg	B220	Inductive, NALT	Effector, NP	ILFs	PPs	Effector, i-LP
IgM	+	46.75 ± 0.19	0.53 ± 0.1	49.6 ± 5.8	63.9 ± 4.9	0.31 ± 0.11
IgA	+	0.79 ± 0.59	0.06 ± 0.01	11.3 ± 3.1	8.2 ± 1.7	0.06 ± 0.04
IgA	−	0.20 ± 0.14	7.69 ± 1.2	1.9 ± 0.8	0.5 ± 0.2	21.7 ± 5.3

[a] MCs were isolated from the organized inductive and effector tissues of the respiratory and intestinal tracts for flow cytometric analysis with fluorochrome-conjugated mAbs anti-μ, anti-α, and PE-conjugated mAb anti-B220. The percentages of the B cell subset indicated in the *left column* in various tissues are shown in each row. The data are presented as the mean ± SD from three separate experiments. sIg, surface Ig.

1 μg of cholera toxin (CT; Sigma-Aldrich, St. Louis, MO), a potent immunogen with strong adjuvanticity (17), once a week for 3 consecutive wk (16).

Cell preparation

Mononuclear cells (MCs) were isolated from spleen, PPs, NP, and NALT as described previously (16, 18). MCs from spleen and NALT were obtained using a mechanical dissociation procedure (16, 18). MCs from PPs and NP were prepared using the enzymatic dissociation protocol with collagenase D (Roche, Mannheim, Germany) (16, 18). MCs from ILFs were prepared as described previously (12). In brief, the small intestine was opened longitudinally along the mesenteric wall. After removal of mucus and feces, an intestinal fragment ~3 cm in length was pasted on a culture dish. Under a transillumination stereomicroscope (TH3; Olympus, Tokyo, Japan), a tiny fragment of the small intestine containing a single ILF was amputated by a sharpened needle. After removal of PPs and ILFs, i-LP lymphocytes were isolated from the small intestine by the enzymatic dissociation procedure with collagenase D (Roche) (18).

Flow cytometry

FITC-conjugated anti-mouse IgA and IgM, and R-PE-conjugated anti-B220 were used for staining (BD PharMingen, San Jose, CA) (18). MCs isolated from different tissues were preincubated with Fc Block (2 μg/ml; BD PharMingen) before fluorochrome-conjugated mAbs. MCs were then incubated with optimal concentrations of FITC-conjugated anti-mouse IgA (2 μg/ml) or IgM (2 μg/ml) together with PE-conjugated anti-B220 (2 μg/ml). Flow cytometric analysis was then performed using FACSCalibur (BD Biosciences, San Jose, CA) (18).

Immunohistochemical analysis

Vertically oriented sections of the small intestine that included PPs and/or ILFs as well as NP and NALT were prepared as previously described (2, 12). Briefly, the small intestine was longitudinally opened along the mesenteric wall, rolled up, and frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen. Serial frozen sections were then incubated with FITC-conjugated anti-IgA (2 μg/ml) or FITC-conjugated anti-IgM (2 μg/ml) and PE-conjugated anti-B220/CD45R (2 μg/ml; all from BD PharMingen) for 1 h at room temperature. The slides were then examined using confocal microscopy (Bio-Rad, Hercules, CA). After confocal fluorescence microscopic analysis, the sections were counterstained with H&E. The sections used for examination were prepared from at least three individual mice.

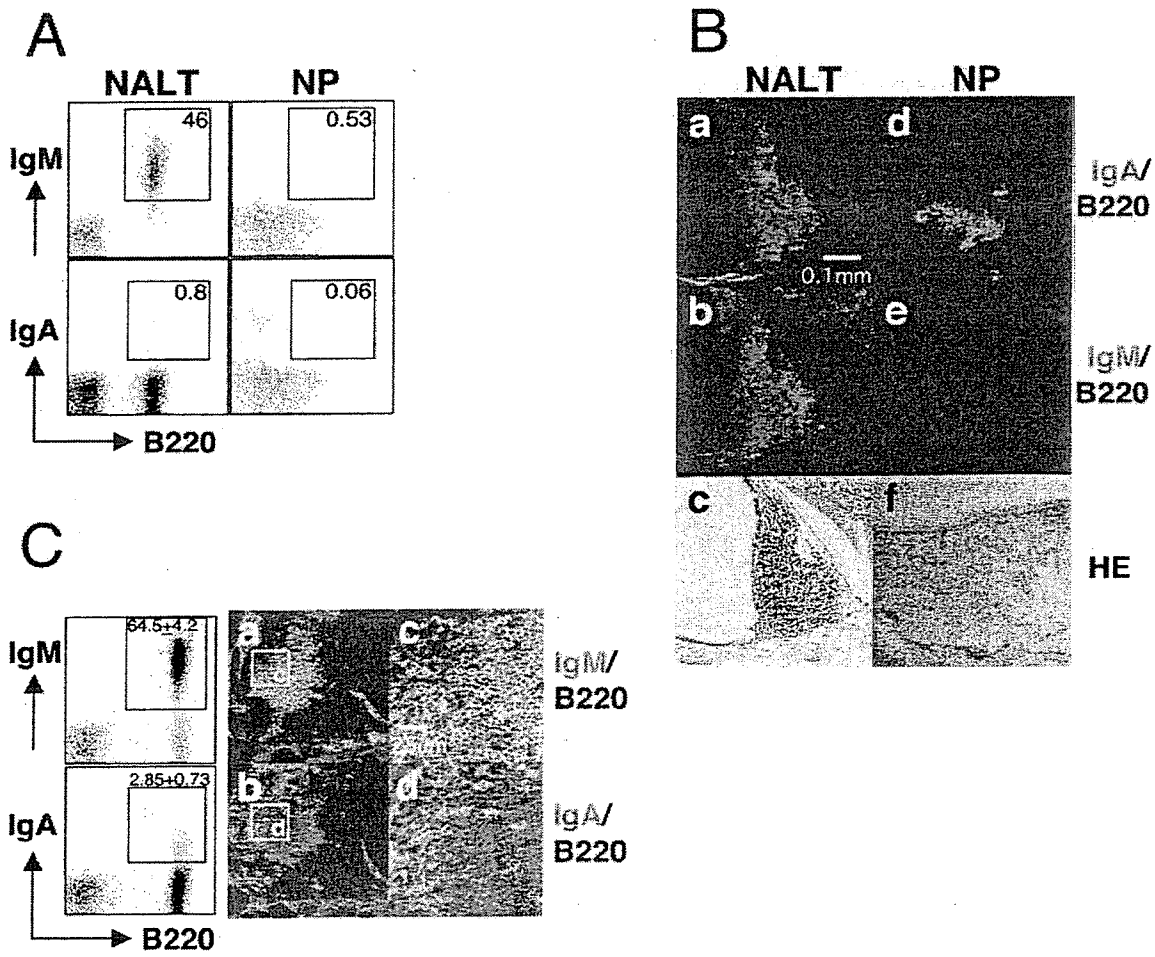

FIGURE 2. Selective localization of IgM^+B220^+ cells in NALT, but not in NP. *A*, MCs isolated from NALT and NP were stained with a panel of mAbs specific for IgM, IgA, and B220. *B*, Consecutive tissue sections were stained with FITC-conjugated anti-IgA (*a* and *d*), anti-IgM (*b* and *e*), and PE-conjugated anti-B220 (*a*, *b*, *d*, and *e*). Samples of each tissue were also stained with HE (*c* and *f*). Cells expressing IgA or IgM (green) as well as those expressing B220 (red) were found only in NALT (*a* and *b*). The numbers of double-positive cells (yellow) of IgA or IgM and B220 were negligible (*d* and *e*), although IgA^+B220^- blasts and plasma cells were plentiful (*d*). *C*, IgA^+ $B220^+$ B cells increased in NALT after nasal immunization with CT. MCs isolated from NALT were stained with a panel of mAbs specific for IgM, IgA, and B220. Consecutive tissue sections were stained with FITC-conjugated anti-IgM and PE-conjugated anti-B220 (*a*) or FITC-conjugated anti-IgA and PE-conjugated anti-B220 (*b*). Higher magnification of sections *a* and *b* are shown in *c* and *d*, respectively. Double-positive cells (yellow) for IgA (green) and B220 (red) were increased in NALT (*C-b* or *C-d*) after nasal immunization with CT (0.8–2.9%). In contrast, double-positive cells (yellow) for IgM (green) and B220 (red) were found in both naive and immunized NALT (*B-b* and *C-a* or *C-c*). These sections were analyzed by confocal microscopy using ×35 (*B*, *C-a*, and *C-b*) and ×200 (*C-c* and *C-d*) magnifications. Results are representative of three separate experiments.

RT-PCR

Total RNA isolated from mouse tissues was extracted, following the manufacturer's instructions, using TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared by reverse transcriptase (Invitrogen) with oligo(dT) primer. AID transcripts, αCTs, Iμ-Cα, and β-actin transcripts were amplified as described previously (9–11). The oligonucleotide primers specific for AID transcripts (5′-GGCTGAGGTTAGGGTTCCATCTCAG-3′ and 5′-GAGG GAGTCAAGAAAGTCACGCTGGA-3′), Iα-Cμ circle transcripts (5′-CCAGGCATGGTTGAGATAGAGATAG-3′ and 5′-AATGGTGCTGGG CAGGAAGT-3′), Iμ-Cα transcripts (5′-CTCTGGCCCTGCTTATTGT TG-3′ and 5′-GAGCTGGTGGGAGTGTCAGTG-3′), and β-actin transcripts (5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGC TCAGTAACAGTCCG-3′) were prepared according to previously described methods (9–11). The experiments were conducted on three separate occasions.

Results

Focal accumulation of IgM^+B220^+ and IgA^+B220^+ cells in organized GALT structures, but not diffuse effector tissues

To investigate the exact class-switching sites in different parts of the wall of the mouse small intestine, we initially examined and compared the localized presence of IgM^+B220^+ B cells, which are considered to be prerequisite for class switching recombination (19, 20). To accomplish this goal, careful separation of the organized lymphoid tissue and the diffuse effector tissue was required. GALT, including PPs and especially ILFs, was carefully identified and removed. After the removal of GALT, but before enzymatic dissociation, the intestine was further re-examined, using microscopic analysis, to ensure and confirm the complete removal of organized lymphoid tissue. Screening for IgM^+B220^+ B cells revealed their presence in ILFs and PPs, but their almost complete absence from i-LP (Fig. 1*A* and Table I). Furthermore, because IgA^+B220^+ B cells are believed to result from recent class switching involving the expression of CSR molecules such as AID and looped-out circular DNA (11), we next screened for the presence of IgA^+B220^+ B cells in these different intestinal mucosa-associated tissues. Although FACS analysis revealed the presence of IgA^+B220^+ B cells in ILFs and PPs, hardly any were detected in i-LP (Fig. 1*A* and Table I). Furthermore, the preferential localization of both IgM^+B220^+ cells and IgA^+B220^+ B cells in the organized lymphoid tissues was also revealed by immunohistochemical analysis using fluorescence confocal microscopy (Fig. 1*B*). By contrast, both populations were absent in diffuse i-LP regions. Instead, larger numbers of IgA^+B220^- B cells, corresponding to the blast and plasma stages, were found in diffuse effector tissues (Table I). Taken together, these observations suggest that the isotype class switching of B cells from μ- to α-chains occurs selectively in the organized lymphoid structure of ILFs and PPs, but not in the diffuse effector tissues of i-LP of the small intestine.

In the upper respiratory tract, IgM^+B220^+ and IgA^+B220^+ B cells are present in NALT, but not in NP

In the generation of IgA-committed B cells, NALT has been shown to be as important an inductive site as GALT (1, 15, 16). When similar evaluation of the respiratory mucosal immune system for IgM^+B220^+ B cell presence was conducted, the tissue localization pattern was similar to that we described for the intestinal tract. Thus, IgM^+B220^+ B cells were preferentially localized in the organized NALT, but were virtually absent from the diffuse effector tissues of the NP (Fig. 2, *B-b* or *B-e*, and Table I). In contrast to PPs and ILFs, extremely few IgA^+B220^+ B cells were found in NALT (Fig. 2*B-a* and Table I). Ag stimulation by bacterial flora of the upper respiratory tract is weaker than that by gut flora surrounding GALT, and we hypothesized that this was the reason for the lack of IgA^+B220^+ B cells in NALT. To test this hypothesis we performed nasal immunization with CT, which is known to possess a strong antigenicity with potent mucosal adjuvant activity (17). After nasal immunization, the presence of IgA^+B220^+ B cells increased in NALT (Fig. 2*C* and Table II). The formation of GCs and the accumulation of IgA^+B220^+ B cells were also seen in the NALT of these nasally immunized mice (Fig. 2*C*). These results demonstrate that in the upper respiratory tract, IgA isotype class switching occurs in the organized NALT, but not in the diffuse lamina propria of the NP.

Table II. *Influence of nasal CT on the development of IgA^+ B cells in NALT*[a]

B Cell Subsets		Nasal Immunization	
sIg	B220	Before	After
IgM	+	54.8 ± 6.90	64.5 ± 4.2
IgA	+	0.93 ± 0.72	2.85 ± 0.73
IgA	−	0.04 ± 0.02	0.48 ± 0.09

[a] After nasal immunization with CT, which is known to possess a strong antigenicity with potent mucosal adjuvant activity, MCs were isolated from NALT and then analyzed by flow cytometry with fluorochrome-conjugated mAbs anti-μ, anti-α, or PE-conjugated mAb anti-B220. Percentages of the B cell subset indicated in the *left column* in NALT before and after nasal immunization with CT are shown in each row. The data are presented as the mean ± SD from three separate experiments. sIg, surface Ig.

Expression of CSR-associated mRNA observed in the organized MALT structure, but not in diffuse effector tissues

To test for CSR from the μ-chain to the α-chain at the molecular level, three molecular markers, AID, αCTs, and Iμ-Cα, were selected. AID was essential for CSR and completely regulated stimulated B cells undergoing class switching (9–11). αCTs were produced from circular DNA that is looped out and lost after CSR (9–11). Iμ-Cα transcripts were produced from α germline transcripts after looping out of αCTs (9–11). Because the expression of AID and αCTs is strictly up-regulated during and is quickly down-regulated after isotype class switching, such expression is considered to characterize the class switching of B cells from μ to α (10). Meanwhile, Iμ-Cα transcripts were seen to be expressed immediately after completion of IgA-specific CSR (11). To further confirm that IgA isotype class switching occurs in vivo at the organized MALT, we next used RT-PCR analysis to test for the expression of CSR-associated molecules, including AID, αCTs,

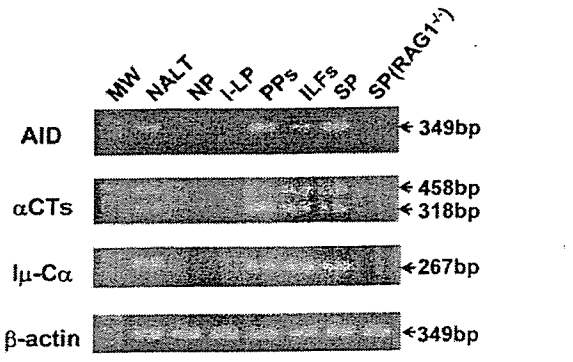

FIGURE 3. Preferential presence of AID, αCT, and Iμ-Cα transcripts in the organized lymphoid tissues of NALT, PPs, and ILFs, but not in the diffuse effector tissues of NP and i-LP. RT-PCR analysis revealed AID, αCT, and Iμ-Cα transcripts in NALT, PPs, and ILFs, but not in i-LP and NP. As a negative control for RT-PCR, the total RNA of MCs isolated from spleen of recombinase-activating gene-1(RAG1)$^{-/-}$ mice was used. The experiments were conducted on three separate occasions.

and Iμ-Cα transcripts, in the organized lymphoid and diffuse effector tissues of the gastrointestinal and respiratory tracts. The results were consistent with our finding of histological localization of IgM^+B220^+ B cells and IgA^+B220^+ B cells (Figs. 1 and 2, and Tables I and II). mRNA expressions of AID, αCTs, and Iμ-Cα transcripts were restricted to the organized MALT, such as ILFs, PPs, and NALT, but were not found in the diffuse effector tissues of i-LP and NP (Fig. 3). These results indicate that IgA isotype class switching selectively occurs in the organized MALT, but not in the diffuse effector tissues.

Discussion

PPs have been shown to contain all the cellular and microarchitectural environments (e.g., B cell follicle, including GCs, follicular dendritic cells network, and interfollicular T cell area) needed for the generation of IgA-committed B cells (1). A large number of IgM^+B220^+ B cells in the GCs of PP follicles express AID and undergo the molecular event of μ to α isotype class switching (9). Results have shown that the incubation of IgM^+ IgA^- B cells isolated from PPs together with TGF-β results in the induction of IgA isotype class switching, which leads to the generation of IgM^-IgA^+ B cells (5–8). After generation in PPs, these postswitched IgA^+ B cells (or IgA-committed B cells) migrate to effector tissues, such as i-LP, where they become IgA blast and plasma cells under the influence of IgA-enhancing cytokines IL-5, IL-6, and IL-10 (1). Therefore, it is generally accepted that organized MALT, such as PPs, acting as inductive sites, play a major role in the initiation of the IgA Ab response, while diffuse i-LP tissues provide the effector sites (1). This conjecture was further confirmed by our current results, which show that IgM^+B220^+ B cells capable of undergoing μ to α isotype class switching were exclusively located in PPs and were absent from diffuse i-LP. In contrast, large numbers of IgA blast and plasma cells were found in these diffuse i-LP. Furthermore, evaluation of CSR-associated molecules for the μ to α gene rearrangement revealed that AID, αCTs, and Iμ-Cα transcripts were selectively expressed in PPs with the organized lymphoid structure, but not in the diffuse i-LP.

In addition to testing PPs, we tested ILFs, which we recently characterized as part of the organized GALT on the antimesenteric wall of the mouse small intestine (12). This cell formation is composed of a large B cell area, including GCs. A large fraction of the B cells in ILFs are B-2 cells, similar to those found in PPs (12). In this study our findings further demonstrate that ILFs are a rich source of IgM^+B220^+ B cells that can undergo μ to α CSR. Thus, CSR-associated transcripts of AID, αCT, and Iμ-Cα were present in the mRNA preparation obtained from ILF as well as PP samples. After careful removal of ILFs and PPs, MCs that had been isolated from the diffuse effector tissues of i-LP were evaluated for the IgA-associated CSR-associated transcripts. No AID, αCT, or Iμ-Cα transcripts were found in the diffuse tissue of i-LP. Taken together, these findings indicate that ILFs and PPs, both of which contain B cell follicles with GCs, constitute the organized GALT and behave as key inductive sites for μ to α isotype class switching when IgA-committed B cells are generated.

When we evaluated the presence and population density of IgM^+ B cells and/or IgA^+ B cells in different histological locations of the upper respiratory tract, accumulations of IgM^+B220^+ B cells were always observed only in the organized MALT such as NALT. These cells were absent in the NP, a representative diffuse effector site for the upper respiratory tract (Fig. 2 and Table I). In addition, AID, αCT, and Iμ-Cα transcripts were expressed in NALT, but not in NP, paralleling expression by ILFs and PPs, but not i-LP, in the intestinal tract (Fig. 3). Previously we have shown that NALT B cells belong to a subset of B-2 cells (18). That study also showed that IgA isotype class switching of B-2 cells in the upper respiratory tract occurs only in the organized MALT, such as NALT, and not in the diffuse lamina propria region of the NP.

Comparisons of NALT and GALT (e.g., ILFs and PPs) for the population density of switched IgA^+B220^+ B cells revealed a dramatic difference. Much higher numbers of IgA^+B220^+ B cells were found in GALT (e.g., 6.5–14.4%; Table I) than in NALT (e.g., 0.2–1.4%; Table I). That finding may be explained by the different microbial environments found in NALT and GALT. In comparison with NALT, GALT structures are situated in a part of the gut where there is an enormous load of microbial Ags and mitogens to continuously stimulate immunocompetent cells located in the organized lymphoid tissue for the mucosal immune system. Thus, administration of CT possessing potent immunogenicity and adjuvanticity via the nasal route resulted in a 3- to 10-fold increase in the population (e.g., 2.9 ± 0.7%; Fig. 2). This increase points to the importance of antigenic stimulation in the initiation of IgA class isotype switching for the generation of IgA-committed B cells in NALT. Elsewhere, we have shown that the tissue organogenesis of NALT is also accelerated by nasal exposure to Ag (21).

Acknowledgments

We thank Noriko Kitagaki for her technical help, and the members of the Mucosal Immunology Group of Osaka University for their critical comments. We also thank Misako Hashimoto for her secretarial assistance.

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Role of Gut-Associated Lymphoreticular Tissues in Antigen-Specific Intestinal IgA Immunity[1]

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This study assessed the roles of the postnatal lymphotoxin-β receptor (LTβR)-mediated signals in the gut-associated lymphoreticular tissues of mice for subsequent regulation of Ag-specific intestinal IgA responses. Blockade of LTβR-dependent events by postnatal administration of the fusion protein of LTβR and IgG Fc (LTβR-Ig) reduced both the size and numbers of Peyer's patches (PP) without influencing the PP microarchitecture. Interestingly, inhibition of LTβR-dependent signaling revealed significant reductions in the formation of follicular dendritic cell clusters in mesenteric lymph nodes (MLN). Furthermore, these postnatal signaling events controlled the development of isolated lymphoid follicles (ILF) because treatment with LTβR-Ig eliminated the formation of ILF. LTβR-Ig-treated mice with altered microarchitecture of MLN and lacking ILF were still able to produce significant Ag-specific mucosal IgA responses after oral immunization; however, the levels were significantly lower than those seen in control mice. These results imply the importance of ILF for Ag-specific intestinal immunity. However, mice treated with both TNFR55-Ig and LTβR-Ig in utero, which lack PP and MLN, but retain intact ILF, failed to induce Ag-specific IgA responses after oral immunization. These findings demonstrate that ILF are not essential for induction of intestinal IgA Ab responses to orally administered Ag. Furthermore, the induction of intestinal IgA Ab responses requires the proper maintenance of the MLN microarchitecture, including a follicular dendritic cell network. *The Journal of Immunology,* **2004, 173: 762–769.**

Mucosal surfaces such as those in the gastrointestinal (GI)[3] tract provide a first line of defense against colonization by microbial pathogens (1). This area is protected by the mucosal immune system, which includes a secretory IgA (S-IgA) Ab response. This IgA constitutes greater than 80% of all Ig isotypes produced in mucosa-associated tissues in humans, and S-IgA is induced, transported, and regulated by mechanisms that are completely different from those involved in systemic Ab responses (2). Although the induction of Ag-specific S-IgA Ab responses is known to be dependent upon cognate help provided by $CD4^+$ Th cells in gut-associated lymphoreticular tissues (GALT), the exact site in which this regulatory network is formed for the subsequent development of these IgA Ab responses is only partially understood (2). In this regard, Peyer's patches (PP) have been considered to be the major inductive site for initiation of Ag-specific IgA immune responses in the GI tract (3). It has been shown that Ag-specific IgA precursors from PP repopulate the intestinal lamina propria via the common mucosal immune system and subsequently differentiate into IgA-producing plasma cells (4). Furthermore, the introduction of Ag into surgically constructed intestinal loops with PP resulted in the appearance of Ag-specific IgA Abs after immunization, whereas no mucosal IgA Ab responses were detected in the intestinal loops that lacked PP (5). These studies support the current dogma that PP are a primary inductive site for Ag-specific immune responses in the gut. However, our previous study showed that oral immunization of mice that lack PP resulted in the generation of Ag-specific IgA Ab responses in the GI tract (6), suggesting the existence of a PP-independent pathway for mucosal IgA Ab responses.

TNF, lymphotoxin (LT) α, and LTβ are members of the TNF superfamily, which also include immunoregulatory molecules such as CD30 ligand (L), CD40L, FasL, TRAIL, and LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes) (7–9). Both TNF and LTα can be secreted as homotrimers, and TNF also exists as a cell surface homotrimer. TNF and LTα homotrimers bind and signal via either of two TNF receptors, TNFR55 or TNFR75 (10, 11). In contrast, LTβ is a type II integral membrane protein that complexes with LTα to form membrane-anchored heterotrimers (12, 13). The LTαβ complex binds to a specific receptor termed the LTβR (14). Mice genetically depleted of LTα, LTβ, or LTβR have disrupted PP and lymph node (LN) development (15–18). In contrast, TNFR55-deficient ($TNFR55^{-/-}$) or $TNFR75^{-/-}$ mice retain PP and LN (19–21). Furthermore, genesis of PP and LN was disrupted by in utero blockade of the LTαβ pathway (22, 23). These studies have shown that ligation of the LTβR by LTαβ heterotrimer during gestation is critical for the formation of PP and LN. However, the generation of the mesenteric lymph nodes (MLN) may require TNF/LTα in addition to the LTαβ signaling

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Received for publication January 20, 2004. Accepted for publication May 5, 2004.

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[1] This work was supported by a Grant-in-Aid for Scientific Research (1437681) from Japan Society for the Promotion of Science, a Grant from the Ministry of Education, Culture, Sports, Science and Technology to promote 2001-Multidisciplinary Research Projects, and the Ministry of Health and Labor, Core Research for Engineering, Science, and Technology, Japan Science and Technology Agency of Japan. This work was also supported by U.S. Public Health Service National Institutes of Health Grants AI 18958, AI 43197, and DE 12242.

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[3] Abbreviations used in this paper: GI, gastrointestinal; AFC, Ab-forming cell; CT, cholera toxin; FDC, follicular dendritic cell; GALT, gut-associated lymphoreticular tissues; GC, germinal center; ILF, isolated lymphoid follicles; L, ligand; LN, lymph node; LT, lymphotoxin; MLN, mesenteric lymph nodes; PNA, peanut agglutinin; PP, Peyer's patch; S-IgA, secretory IgA; TT, tetanus toxoid.

0022-1767/04/$02.00

cascade (24). A role in MLN organogenesis has also been postulated for the alternative LTβR-binding ligand termed LIGHT (25).

Our previous study showed that Ag-specific IgA responses could be induced in mice with a deficiency in organized PP (6). Furthermore, recent studies have reported that the LTαβ signaling pathway in the intestinal lamina propria is relevant for the production of IgA in a manner shown to be independent of the presence of PP and MLN (26, 27). These studies imply that the GALT immune system is equipped with multiple pathways, and the well-characterized PP and MLN network is just one form of the induction cascade for IgA responses in the GI tract. To this end, a recent study has demonstrated that, in addition to PP, there are small lymphoid clusters, termed isolated lymphoid follicles (ILF), in the small intestine (28, 29). ILF formation occurs postnatally in response to luminal stimuli, including normal bacterial flora (29, 30). Thus, ILF may be a component of GALT that potentially contribute to intestinal IgA immunity. To test the hypothesis that ILF contribute to the IgA responses, TNF55R- and/or LTβR-mediated signaling pathways were pre- or postnatally manipulated in vivo for the creation of ILF-compromised mice.

Materials and Methods

Mice

C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). These mice were maintained in the experimental facility under pathogen-free conditions in the Nihon University School of Dentistry at Matsudo (Chiba, Japan). The mice were free of bacterial and viral pathogens, as determined by pathogenic and serologic analysis on sentinel mice.

Fusion proteins and treatment protocols

Proteins comprised of the extracellular domain of either murine TNF receptor 55 or LTβR fused to the hinge, CH2, and CH3 domains of human IgG1 (LTβR-Ig and TNFR55-Ig, respectively) were used in our studies, as described elsewhere (22, 31, 32). Young adult mice (6 wk old) were injected i.p. with 100 μg of TNFR55-Ig or LTβR-Ig at weekly intervals during the immunization period from day −7 to day +14 relative to the day of immunization. Pharmacokinetic analysis showed that the $t_{1/2}$ of LTβR-Ig was 4–6 days, and this treatment dose provided 6–11 μg/ml fusion protein in the serum. In some experiments, pregnant mice were injected i.v. with 200 μg of both TNFR-55-Ig and LTβR-Ig on gestational days 13 and 16, as described previously, with minor modifications (6, 22).

Immunization

A vaccine grade of tetanus toxoid (TT) was kindly provided by Y. Higashi (Biken Foundation, of Osaka University, Suita, Osaka, Japan). For oral immunization, mice were deprived of food for 2 h and then given a solution of sodium bicarbonate to neutralize stomach acidity before oral immunization (33). Thirty minutes later, these mice were orally immunized by gastric intubation with 250 μg of TT in the presence of 10 μg of cholera toxin (CT) as mucosal adjuvant (34). This oral immunization procedure was conducted on days 0, 7, and 14.

Immunohistochemical analysis

Freshly isolated MLN and PP were rapidly frozen in OCT embedding medium (Tissue-Tek, Elkhart, IN) and stored at minus 80°C until processing. Cryostat sections (6 μm) were fixed in ice-cold acetone for 10 min, dried, and preblocked with anti-CD16/CD32 Fc block (BD Pharmingen, San Diego, CA) in PBS. Cells were stained with FITC-conjugated anti-B220 mAb (BD Pharmingen) and PE anti-CD3 mAb (BD Pharmingen). Reactivity with peanut agglutinin (PNA) was demonstrated using biotinylated PNA (Vector Laboratories, Burlingame, CA), followed by streptavidin-PE. Staining of sections for follicular dendritic cell (FDC) clusters used biotinylated CR1 (BD Pharmingen), followed by streptavidin PE. IgA-containing cells were visualized by FITC-labeled anti-mouse IgA mAb (BD Pharmingen). The sections were mounted and viewed under ×100 optics and a dual red/green filter. Each of the images was analyzed with Photoshop (Adobe Systems, San Jose, CA) in a consistent manner, followed by overlying of the green and red images in the screen mode (MicroRadience AG-2; Bio-Rad, Hercules, CA). The final images are representative of these sections.

Determination of numbers of ILF

The small intestine was divided into four equal parts distal from the pylorus, and segments (2 cm long) were collected from each part. The tissue segments were fixed in 4% paraformaldehyde and embedded in paraffin. The segments were sectioned consecutively at 6 μm and stained with H&E for counting the numbers of ILF. In some experiments, the segments were frozen in OCT embedding medium and the cryostat sections were stained with PE-conjugated anti-B220 mAb (BD Pharmingen).

Detection of Ag-specific Ab isotype responses

Serum and fecal extracts were obtained, and Ag-specific Ab titers were determined by an ELISA, as described elsewhere (33). Briefly, plates were coated with TT (5 μg/ml) and blocked with 10% goat serum. Analyses were performed in duplicate. Following 4 h of incubation, the plates were washed, and peroxidase-labeled goat anti-mouse μ, γ, or α H chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to appropriate wells. Finally, ABTS with H_2O_2 (Moss, Pasadena, CA) was added for color development. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution, which gave an OD at 414 nm of 0.1 greater than background after 15 min of incubation.

ELISPOT for assessment of Ab-forming cells (AFCs)

Single cell suspensions were obtained from intestinal lamina propria, as previously described (33). The mononuclear cells were obtained at the interface of the 40 and 75% layers of a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ). To assess numbers of total and Ag-specific AFCs, an ELISPOT assay was performed, as previously described (35). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with goat anti-mouse Ig Ab (2 μg/ml) (Southern Biotechnology Associates) or TT (5 μg/ml), incubated for 20 h at 4°C, and then washed extensively and blocked with 10% goat serum. The blocking solution was discarded, and lymphoid cell suspensions at various dilutions were added to wells and were incubated for 4 h at 37°C in 5% CO_2 in moist air. The detection Abs consisted of goat HRP-conjugated anti-mouse α or γ H chain-specific Abs (Southern Biotechnology Associates). Following overnight incubation, the plates were washed with PBS and developed by addition of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H_2O_2 (Moss) to each well. Plates were incubated at room temperature for 15–20 min and washed with water, and AFCs were counted with the aid of a stereomicroscope (SZH-ILLB; Olympus, Tokyo, Japan).

Statistics

The data are expressed as the mean ± SEM and compared using the unpaired Mann-Whitney *U* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for Macintosh computers.

Results

Signaling pathways through TNFR55 and/or LTβR have diverse effects on the maintenance of GALT

We first evaluated the role of TNF/LTα and LTαβ signaling pathways on the maintenance of the fully developed intestinal mucosal immune system, and thus for the induction of Ag-specific mucosal IgA Ab responses. Signaling pathways through TNFR55 or LTβR in adult mice were blocked by introducing murine TNFR55-Ig or LTβR-Ig fusion proteins as soluble receptor decoys. Thus, 6-wk-old mice were injected i.p. with TNFR55-Ig or LTβR-Ig at weekly intervals. Treatment of normal adult mice with TNFR55-Ig or LTβR-Ig had no effect on the numbers or size of MLN (data not shown). Histological analysis showed that the MLN of TNFR55-Ig- as well as control-Ig-treated mice had organized and distinct B cell follicles (Fig. 1, *A* and *E*). In contrast, when mice were treated with LTβR-Ig, the follicles of the MLN were disrupted (Fig. 1*I*). The MLN of TNFR55-Ig- as well as control-Ig-treated mice retained a distinct segregation of lymphocytes into a superficial cortical B cell zone and a deep cortical T cell zone (Fig. 1, *B* and *F*). In contrast, although T and B cell areas still appeared to be segregated in LTβR-Ig-treated mice, the border of these areas became indistinct and had more diffuse edges (Fig. 1*J*).

Additional abnormalities of the MLN microarchitecture were observed in fusion protein-treated adult mice. Control, human