through the consumption of foodstuffs. For instance, probiotic bacteria digest exogenous and endogenous materials (e.g., fibers and mucins), and the broken down products affect the host immune system [40]. A recent study reported that short-chain fatty acids produced from fiber by commensal bacteria are required for the normal resolution of inflammatory responses through G-protein–coupled receptor 43 [41•].

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

Dietary Materials and Milk in the Development of Food Allergy

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

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

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

Conclusions

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

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- • Of importance
- •• Of major importance

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Indigenous opportunistic bacteria inhabit mammalian gut-associated lymphoid tissues and share a mucosal antibody-mediated symbiosis

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The indigenous bacteria create natural cohabitation niches together with mucosal Abs in the gastrointestinal (GI) tract. Here we report that opportunistic bacteria, largely *Alcaligenes* species, specifically inhabit host Peyer's patches (PPs) and isolated lymphoid follicles, with the associated preferential induction of antigen-specific mucosal IgA Abs in the GI tract. *Alcaligenes* were identified as the dominant bacteria on the interior of PPs from naïve, specific-pathogen-free but not from germ-free mice. Oral transfer of intratissue uncultured *Alcaligenes* into germ-free mice resulted in the presence of *Alcaligenes* inside the PPs of recipients. This result was further supported by the induction of antigen-specific Ab-producing cells in the mucosal (e.g., PPs) but not systemic compartment (e.g., spleen). The preferential presence of *Alcaligenes* inside PPs and the associated induction of intestinal secretory IgA Abs were also observed in both monkeys and humans. Localized mucosal Ab-mediated symbiotic immune responses were supported by *Alcaligenes*-stimulated CD11c$^+$ dendritic cells (DCs) producing the Ab-enhancing cytokines TGF-β, B-cell-activating factor belonging to the TNF family, and IL-6 in PPs. These CD11c$^+$ DCs did not migrate beyond the draining mesenteric lymph nodes. In the absence of antigen-specific mucosal Abs, the presence of *Alcaligenes* in PPs was greatly diminished. Thus, indigenous opportunistic bacteria uniquely inhabit PPs, leading to PP-DCs-initiated, local antigen-specific Ab production; this may involve the creation of an optimal symbiotic environment on the interior of the PPs.

Alcaligenes | intratissue habitation | Peyer's patch

The intestine is most frequently exposed to a huge number and a wide variety of environmental antigens, including bacteria and food products. As a result, indigenous bacteria create appropriate homeostatic conditions for physiologic processes such as the production of vitamin K and the metabolism of indigestible dietary carbohydrates and polysaccharides (1). In addition to nutritional mutualism, microbial stimulation is required for full maturation of the host immune system, including intestinal secretory IgA (SIgA) production (2). It was demonstrated that germ-free (GF) mice have an immature mucosal immune system, including hypoplastic Peyer's patches (PPs) and diminished numbers of IgA-producing cells and CD4$^+$ T cells (3). Both naturally occurring and acquired Abs in the intestine are of the IgA isotype. SIgA Abs recognize either T cell-independent or -dependent forms of antigens, which may limit the adherence of commensal bacteria to epithelial cells and prevent their penetration into deeper mucosal and systemic lymphoid tissues (4, 5).

Our current understanding is that commensal bacteria in the lumen and intestinal IgA together create natural cohabitation niches in the gastrointestinal (GI) tract (6). However, the nature and location of these cohabitation niches remain to be elucidated because more than 90% of the intestinal microbes have not been cultured. This limits the ability to perform detailed immunologic and bacteriologic analyses of the cohabitation mechanism between the host immune system and commensal bacteria. However, recent advances in the 16S rRNA gene clone library analysis technique have made it possible to study the composition of symbiotic bacteria in the GI tract (7, 8) and thus allow us to understand the molecular and cell biology of bilateral interactions between the mucosal immune system and the intestinal microbiota.

PPs are an example of well-characterized gut-associated lymphoid tissue and contain a wide variety of immunocompetent cells, including dendritic cells (DCs), macrophages, and B and T cells. The tissues continuously take up gut luminal antigens through M cells, including both beneficial and undesired antigens, and initiate antigen-specific immune responses in the host. The numbers of PPs range from 8 to 10 in the murine, and up to 200 in the human, small intestine (4). In a previous study of the interactions between the GI commensal bacteria and mucosal Ab production, luminal bacteria (e.g., *Enterobacter cloacae*) were shown to be taken up by CD11c$^+$ DCs in the PPs (PP-DCs); this led to the development of the intestinal IgA immune system (9).

Here, we tested the hypothesis that PPs, a major inductive and regulatory site for mucosal immunity (4) and also the entry site for luminal antigens such as indigenous bacteria (9), are one of the intratissue cohabitation niches of the intestinal microbiota necessary for the development of the mucosal immune system. This intratissue colonization may create a state of symbiosis with instructive environmental antigens on the interior of the PPs.

Results

Presence of Indigenous Opportunistic Bacteria on the Interior of PPs. To determine the bacterial composition at the surface and on the interior of PPs in naïve, specific-pathogen-free (SPF) mice, we

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The authors declare no conflict of interest.

Data deposition: The nucleotide sequences reported in this study have been deposited in the International Nucleotide Sequence Database (accession nos. AB453241–AB453250).

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first used the 16S rRNA gene clone library method. Consistent with a previous report (10), segmented filamentous bacteria were the predominant species detected on the surface of the follicle-associated epithelium covering PPs (Fig. 1*A*). In contrast, several species of indigenous microbiota, including *Alcaligenes* spp., *Ochrobactrum* spp., *Serratia* spp., and *Burkholderia* spp., were detected on the interior of PPs. Of these, *Alcaligenes*, which are opportunistic bacteria (11), were dominant (72%; Fig. 1*A*).

To confirm the presence and localization of *Alcaligenes* on the interior of PPs, we next performed a whole-mount FISH analysis to identify the bacterial distribution in this tissue (12). The microbial cells were visualized by three distinct probes used in several previous studies (12–14) (Table S1). EUB338 is routinely used for detecting bacterial species in an indiscriminate manner (12). ALBO34a is a specific probe for *Alcaligenes* and *Bordetella* (13), and BPA is for *Alcaligenes*, *Burkholderia*, and *Comamonas* (14). Thus, *Alcaligenes* are identified as ALBO34a and BPA double-positive cells.

Consistent with the 16S rRNA analysis (Fig. 1*A*), EUB338-positive bacteria morphologically similar to segmented filamentous bacteria were observed over the entire surface area of PPs covered by wheat germ agglutinin positive (WGA$^+$) epithelial cells (Fig. 1*B*). ALBO34a and BPA double-positive *Alcaligenes* were detected on the interior of PPs, where WGA$^+$ epithelial cells were not observed (Fig. 1*B*). Sequential analysis through the *z* axis convincingly showed that *Alcaligenes* were present on the interior of PPs (Movie S1). We also confirmed the presence of *Alcaligenes* by the PCR method in a separate study using the 16S rRNA-gene-targeted group-specific PCR primers for *Alcaligenes*.

In contrast to the preferential localization of *Alcaligenes* in PPs, this species was essentially absent in the diffuse lamina propria (LP) region of the small intestine (Fig. 1*B*), whereas EUB338-positive bacteria were scattered throughout the surface layer of the LP (Fig. S1*A*). Thus, although some antigen-sampling cells [e.g., villous M cells (15) and epithelial DCs (16)] are located in the epithelium covering the more diffuse LP region, it seems that antigen-sampling M cells and DCs in the follicle-associated epithelium of PPs are responsible for the entry of *Alcaligenes*. Furthermore, the presence of *Alcaligenes* inside PPs was demonstrated to be a common feature by the characterization of different species of mice housed in various SPF-maintained experimental animal facilities (Fig. S1*B*). These findings suggest a possibility that commensal bacteria live within the tissues of the organized lymphoid structures associated with the GI tract.

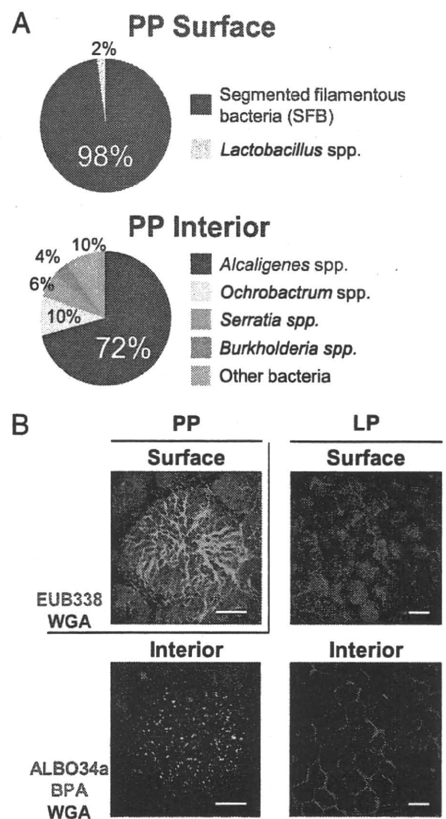

Fig. 1. Microbial distribution in the GI immune compartment. (*A*) Microbial composition at the surface and on the interior of PPs was examined by 16S rRNA gene clone library analysis. (*B*) The presence of *Alcaligenes* was visually analyzed by whole-mount FISH at the surface and on the interior of PPs and LP. Data are representative of five independent experiments. [Scale bars, 100 μm (PP), 150 μm (LP).]

Alcaligenes-Ingested PP-DCs Migrate into Mesenteric Lymph Nodes but not Spleen. We next investigated the fate of *Alcaligenes* inhabiting PPs, and particularly their interactions with mucosal immunocompetent cells. When the microbial populations within DCs purified from different tissues were characterized by the 16S rRNA analysis, *Alcaligenes* were detected within PP-DCs and mesenteric lymph node (MLN) DCs (Fig. 2*A*) but not splenic DCs (Fig. S2). Our findings support the presence of a restricted PP-MLN axis for migration of DCs that have taken up indigenous microbiota and suggest that MLNs act as reinforcement to help prevent intrusions

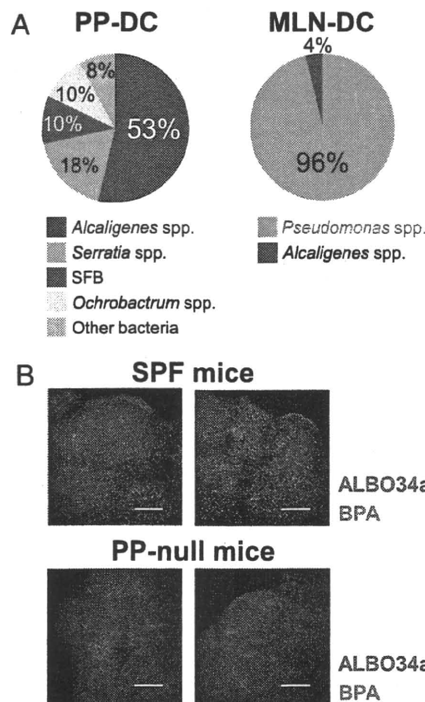

Fig. 2. PP-MLN migration axis for *Alcaligenes*-ingested GI tract DCs. (*A*) CD11c$^+$ DCs were isolated from the PPs and MLNs. Bacterial composition was determined by 16S rRNA gene clone library analysis. (*B*) Whole-mount FISH was performed to detect *Alcaligenes* (yellow) in the MLNs of PP-intact and PP-null mice. The confocal images were sequentially captured at 20-μm intervals along the *z* axis. Data are representative of five independent experiments. (Scale bars, 300 μm.)

by indigenous microbiota into the systemic compartment (17). By using FISH analysis, we also found substantial numbers of *Alcaligenes* in the MLNs of SPF mice (Fig. 2*B*).

To investigate whether PP-DCs are the main source of MLN-DCs harboring *Alcaligenes*, PP-null mice were generated by in utero treatment with an anti-IL-7 receptor α chain mAb (18). In PP-null mice, negligible numbers of *Alcaligenes* were detected in their MLNs (Fig. 2*B*); these bacteria presumably originated from isolated lymphoid follicles (ILFs) (Fig. S1*C* and Movie S2), which resemble PPs and still develop in PP-null mice (19). This result was identical to previous reports showing that PPs are the major sites for uptake of orally inoculated bacteria and the subsequent induction of host immune responses (e.g., *Salmonella typhimurium* and *Helicobacter pylori*) (20, 21).

Preferential Induction of *Alcaligenes*-Specific Mucosal Ab Responses for the Establishment of Symbiosis. To elucidate whether the intratissue presence of *Alcaligenes* and their uptake by PP-DCs affect intestinal mucosal Ab responses, we next examined IgA Ab responses to *Alcaligenes* because IgA is the major isotype of mucosal Abs (4). We used *Alcaligenes faecalis* subsp. *faecalis* NBRC (National Institute of Technology and Evaluation Biological Resource Center) 13111^T, which was the predominant species in the PPs (Fig. S3*A*), for the analysis of antigen-specific immune responses. Substantial amounts of *Alcaligenes*-specific IgA Abs were detected in the feces of SPF mice, whereas GF mice failed to produce this isotype of antigen-specific Abs (Fig. 3*A*, *Left*). No serum IgG Abs specific for *Alcaligenes* were seen in either SPF or GF mice (Fig. 3*A*, *Right*). This result reflected the localization of *Alcaligenes* in PPs, a major mucosal Ab-inductive lymphoid tissue, and not spleen, where systemic IgG Ab responses predominate (Fig. 1 and Fig. S2).

In agreement with this finding, an enzyme-linked immunospot (ELISPOT) assay showed that naïve, SPF mice possessed *Alcaligenes*-specific IgA Ab-forming cells (AFCs) in their intestinal compartments, including PPs and the LP region, but not in the spleen (Table 1). Additionally, no *Alcaligenes*-specific IgG-AFCs were seen in MLNs or spleen (Table 1). *Alcaligenes*-specific IgA-AFCs were more commonly observed in the PPs than in the LP region: more than 2% of IgA-AFCs in the PPs were reactive to *Alcaligenes*, whereas only approximately 0.5% of IgA-AFCs in the LP were specific for *Alcaligenes* (Table 1). This tissue-specific pattern of *Alcaligenes*-specific IgA-AFCs was further confirmed by FACS analysis using GFP-*Alcaligenes* (Fig. S3*B*): 5.3% of IgA-positive B cells (including 2.3% of IgA plasmablasts) were specific for *Alcaligenes* in the PPs, whereas only 1.1% of IgA-positive B cells in the LP were specific for this bacterium (Fig. S3*B*). In addition, when we examined LP-homing properties of local IgA class-switched (or IgA committed) B cells in PPs, *Alcaligenes*-specific IgA^+ B cells expressed fewer gut-homing receptors (α4β7, CCR9, and CCR10) than the rest of the PP-IgA^+ B cells (Fig. S3*C*). Therefore, *Alcaligenes*-specific IgA-committed B cells most likely remained in PPs, which accounted for the presence of elevated *Alcaligenes*-specific IgA-AFCs in PPs compared with LP.

Some intestinal IgA Abs are derived from B1 B cells and recognize T cell-independent antigens commonly expressed by commensal bacteria. Thus, it is possible that *Alcaligenes*-specific IgA Abs show some cross-reactivity with other commensal bacteria. We tested this possibility by FACS analysis and found that *Alcaligenes*-specific Abs did not cross-react with other bacteria (e.g., *Escherichia coli*; Fig. S4*A*). This view was further supported by the analysis of *Alcaligenes*-specific IgA mAb (#3E-12A-6D-3G) developed by fusion of B cells from the PPs of SPF mice. This mAb did not cross-react with *E. coli*. In addition, impaired intestinal IgA Ab responses to *Alcaligenes* were noted in $TCR\beta^{-/-}\ \delta^{-/-}$ mice (Fig. S4*B*). These data suggest that *Alcaligenes*-specific IgA Abs are mostly derived from B2 B cells producing T cell-dependent, antigen-specific Abs. This agrees with the evidence that PPs are major sites for the induction of intestinal mucosal Ab responses to T cell-dependent microbial antigens regardless of whether the microbes are commensal or pathogenic (4).

Although PPs are thought to play a major role in the induction of IgA-committed B cells and plasmablasts, but not plasma cells (4), these data suggest that a large part of *Alcaligenes*-specific fecal IgA Abs are derived from PP IgA-producing cells in a T cell-dependent manner. In fact, markedly decreased levels of anti-*Alcaligenes* fecal IgA Abs were seen in PP-null mice (Fig. S4*C*). These findings are in agreement with previous reports demonstrating that PP-DCs are involved not only in the class-switching of IgM^+ B cells to IgA^+ ones and the determination of gut-tropism via retinoic acid synthesis (22, 23), but also in regulating IgA secretion in the PPs through the stimulation signal provided by the Ab-enhancing cytokine IL-6 (24). We examined IL-6 production by PP cells from GF mice after treatment with *Alcaligenes* and found that *Alcaligenes* induced mainly PP-DCs to produce substantial levels of IL-6 (Fig. S5*A*). When PP-DCs were isolated from WT mice and cocultured with *Alcaligenes*, the synthesis of the IgA isotype-switching cytokines TGF-β and B-cell-activating factor belonging to the TNF family (BAFF) were also elevated in addition to IgA-enhancing cytokine IL-6 (Fig. S5*B*).

Taken together, these findings suggest that mucosal Abs, including locally produced, antigen-specific IgA Abs, may play a critical role in the intratissue cohabitation of *Alcaligenes* in PPs. Supporting this view, *Alcaligenes* numbers were much lower in the PPs of CBA/N *xid* mice, which exhibit a B cell defect, than in WT mice (Fig. 3*B* and Fig. S6*A*). Further, *Alcaligenes* levels tended to be lower also in PPs of IgA-deficient mice, although no statistically significant differences were observed (Fig. S6*B*). Because the IgA-deficient condition did not lead to the complete removal of PP intratissue *Alcaligenesis*, it is also possible that *Alcaligenes*-

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Table 1. Induction of *Alcaligenes*-specific and total AFCs in *Alcaligenes*-associated ex-GF mice

	SPF mice			*Alcaligenes*-associated mice		
Variable	A (Anti-*Alcaligenes*)	B (Total)	A/B × 100 (%)	A (Anti-*Alcaligenes*)	B (Total)	A/B × 100 (%)
IgA-AFCs/10^5 lymphocytes						
PP	28 ± 15	1,304 ± 364	2.10 ± 0.83	10 ± 5	625 ± 307	1.68 ± 0.46
LP	52 ± 12	9,750 ± 3,350	0.57 ± 0.19	12 ± 9	3,133 ± 1,087	0.32 ± 0.20
MLN	2 ± 1	221 ± 64	0.63 ± 0.51	0	20 ± 6	0
Spleen	0	36 ± 8	0	0	15 ± 5	0
IgG-AFCs/10^5 lymphocytes						
MLN	0	13 ± 7	0	0	10 ± 5	0
Spleen	0	15 ± 8	0	1 ± 1	40 ± 18	0.77 ± 1.72

Alcaligenes-specific and total AFCs in SPF and the *Alcaligenes*-associated ex-GF mice were enumerated by ELISPOT assay. Data are expressed as means ± SD (n = 6, respectively).

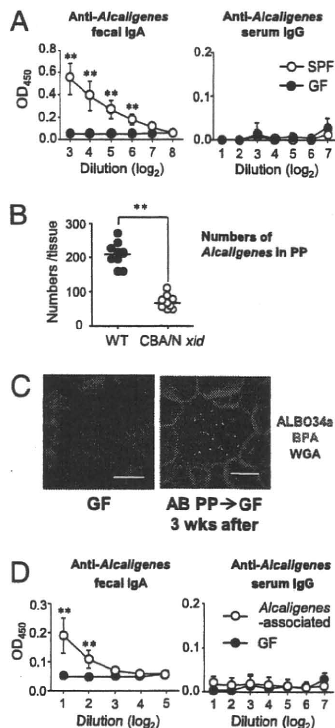

Fig. 3. Preferential induction of *Alcaligenes*-specific mucosal Ab responses in the PPs. (*A*) *Alcaligenes*-specific fecal IgA and serum IgG Ab responses were determined by ELISA. Data are means ± SD (n = 4). (*B*) The numbers of *Alcaligenes* inside PPs were counted in 10 randomly chosen PPs of CBA/N *xid* and WT mice. Data are representative of three independent experiments. Horizontal bar indicates the mean. (*C*) Bacterial distribution on the interior of PPs of GF mice. AB, antibiotic-treated mice. Data are representative of three independent experiments. (Scale bars, 100 µm.) (*D*) *Alcaligenes*-specific fecal IgA and serum IgG Ab responses in the *Alcaligenes*-associated ex-GF mice were measured by ELISA. Data are means ± SD (n = 6). $^{**}P < 0.01$.

specific IgA Abs may not be fully involved in the presence of *Alcaligenes* in PPs. Alternatively, this lack of significant differences may offer another explanation due to the compensation of IgA function by IgM Abs in deficient mice because the numbers of anti-*Alcaligenes* IgM-AFCs was much increased in IgA-deficient mice when compared with WT mice (Fig. S6*C*).

Ability of *Alcaligenes* to Colonize the Interior of PPs. Intratissue cohabitation of *Alcaligenes* in PPs should be addressed formally and directly by the establishment of a gnotobiotic mouse model monoassociated with *Alcaligenes*. The current technology, however, does not permit the isolation and culture of *Alcaligenes* from PPs. Previous studies have shown that *Alcaligenes* have the distinctive feature of being resistant to multiple antibiotics (25, 26), suggesting to us a unique strategy to directly assess the presence of intratissue *Alcaligenes* in PPs. By isolating PPs from antibiotic-treated mice under sterile conditions for the preparation of homogenized tissue and its subsequent oral administration to GF mice, we were able to establish PP-derived, *Alcaligenes*-associated mice. When we examined the antibiotic-treated mice, no bacteria were seen at the intestinal epithelial surface (including the follicle-associated epithelium), whereas *Alcaligenes* were present inside PPs (Fig. S7*A*). Three weeks after oral inoculation, *Alcaligenes* were again noted on the interior of PPs of ex-GF mice (Fig. 3*C*). The colonization of *Alcaligenes* in the PPs of ex-GF mice was further supported by the presence of antigen-specific fecal SIgA but not serum IgG Abs (Fig. 3*D*). A significant increase in antigen-specific IgA- but not IgG-AFCs was also observed in these mice (Table 1). Furthermore, the levels of total IgA were partially increased in the *Alcaligenes*-associated mice (Fig. S7*B*). When we examined PPs of GF mice, the numbers of total IgA-AFCs were 143 ± 45 per 10^5 lymphocytes. On the other hand, the numbers of total IgA-AFCs in PPs isolated from both SPF and the mono-associated mice were 1,304 ± 364 and 625 ± 307, respectively (Table 1). A similar tendency was also seen when total IgA levels were examined in fecal samples taken from monoassociated, GF, and SPF mice (Fig. S7*B*). These findings further suggest that the intratissue habitation of *Alcaligenes* in the PPs may contribute to not only the induction of *Alcaligenes*-specific IgA but also the development of at least a portion of mucosal IgA-associated humoral immunity.

***Alcaligenes* Were Present on the Interior of Monkey and Human PPs.** On the basis of the findings demonstrated by a variety of mouse experiments as described above, we next examined the presence of *Alcaligenes* inside PPs of higher mammals, namely nonhuman primates and humans. This bacterium was observed on the interior of monkey PPs by FISH analysis (Fig. 4*A*, *Left*), and anti-*Alcaligenes* IgA Abs were also detected in the feces of these monkeys (Fig. 4*A*, *Right*). To further demonstrate the intratissue habitation of *Alcaligenes* in monkey PPs, an *Alcaligenes*-specific mAb (#11E-8C-7A, IgM isotype) was developed. Immunohistochemical analysis with *Alcaligenes*-specific mAb #11E-8C-7A showed the presence of this bacterium on the interior of primate PPs (Fig. 4*C*, *Left*). When human PPs were obtained from noninflamed sites of healthy patients who underwent endoscopic biopsy, the intratissue habitation of *Alcaligenes* was demonstrated inside human PPs by FISH

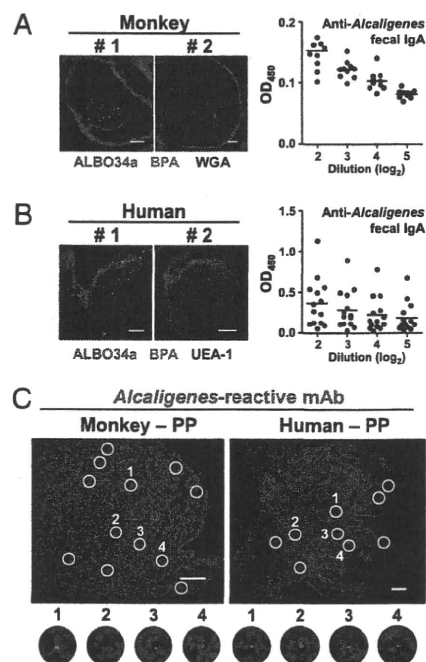

Fig. 4. Intratissue habitation of *Alcaligenes* inside nonhuman primate and human PPs. (*A* and *B*) *Alcaligenes* were detected on the interior of monkey and human PPs by whole-mount FISH (*Left*). *Alcaligenes*-specific fecal IgA Ab responses in monkeys and human were examined by ELISA [*Right*; n = 10 (*A*), n = 14 (B)]. Horizontal bar indicates the mean. (Scale bars, 100 µm.) (*C*) Immunohistochemical analysis was conducted in monkey and human PPs with *Alcaligenes*-reactive #11E-8C-7A mAb and phycoerythrin-labeled anti-mouse IgM Ab. Open circles indicate the presence of *Alcaligenes*. (Scale bars, 100 µm.)

analysis (Fig. 4*B*, *Left*). In addition, anti-*Alcaligenes* fecal IgA Abs were also detected in human fecal samples (Fig. 4*B*, *Right*), consistent with the murine and nonhuman primate studies (Fig. 3*A*, *Left* and Fig. 4*A*, *Right*), The intratissue habitation of *Alcaligenes* in human PPs was further confirmed by the use of *Alcaligenes*-specifc mAb #11E-8C-7A (Fig. 4*C*, *Right*).

Discussion

The present study has revealed a unique aspect of intestinal symbiosis between the host immune system and its indigenous microbiota. In this system some opportunistic bacteria, such as *Alcaligenes*, exploit organized murine mucosal inductive tissues (PPs and ILFs) as their tissue-interior cohabitation niches *in vivo*. The intratissue habitation of *Alcaligenes* was further demonstrated by the analysis of PPs from nonhuman primates and humans. Recently, the microbial composition of mucosa-associated lymphoid tissue (MALT) lymphomas was analyzed by the use of a 16S rRNA method and revealed that *Alcaligenes* were highly detected in those lymphoma tissues (27). This finding also suggests the likelihood that *Alcaligenes* ordinarily inhabit the human mucosal compartment and that the dysregulation of this mutualism in the organized MALT of the host GI tract may contribute to the development of the MALT lymphoma.

The origin of *Alcaligenes* involved in this intratissue colonization remains unknown. *Alcaligenes* are widely present in soil, fresh water, sewage, marine systems, human clinical materials, and the feces of healthy people (11). In this study we attempted to isolate and culture this unique bacterium from PPs of naïve SPF mice, but we unfortunately have not yet developed suitable culture conditions. However, we did confirm that *Alcaligenes faecalis* NBRC 13111^T never entered the PPs after oral inoculation. This may be because *Alcaligenes* can change their morphology, which includes rod-shaped ($0.8–1 \times 1–2$ µm) and coccoid (0.2–1 µm) forms (11). Similarly, *H. pylori* exhibits a coccoid form in the specific environment of the small intestine, which is essential for its selective uptake by PPs and the subsequent induction of antigen-specific and pathogenic $CD4^+$ T cells that cause gastritis (21). Thus, it is possible that a specific form, presumably the coccoid form, of *Alcaligenes* is a prerequisite for its effective transfer into PPs and subsequent establishment of the intratissue cohabitation in the PPs. Supporting this prediction, we detected morphologically small, or presumably coccoid forms of *Alcaligenes* on the surface of the PP (Fig. S8).

An additional observation in the present study was that the numbers of *Alcaligenes* decreased in the absence of B cells and mucosal Abs (Fig. 3*B* and Fig. S6*A*). These results suggest that *Alcaligenes*-specific Abs may play a critical role in the PP tissue colonization by these bacteria. An interesting hypothesis would be that the coccoid form of *Alcaligenes* coated with specific mucosal Abs is selectively taken up by PPs through M cells expressing IgA receptors (28), and formation of the immune complex results in the creation of an appropriate environment for their cohabitation on the interior of PPs.

Another unresolved issue is why *Alcaligenes* exclusively inhabit the PPs. It has already been demonstrated that *Alcaligenes* produce antimicrobial substances inhibiting growth of other bacteria, including multidrug-resistant pathogenic bacteria (29–31). Kalimantacins, antibiotics derived from *Alcaligenes* spp. YL-02632S, were shown to suppress the reproduction of *Staphylococcus* spp., including *Staphylococcus aureus* (29). Further, unique antibacterial compounds produced by *Alcaligenes* spp. FC-88 (30) and M3A (31) were reported to interfere with growth of a wide variety of bacteria, such as *E. coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Thus, the presence of *Alcaligenes* spp. in PPs, the active antigen-sampling site, may be beneficial for the host by eliminating other opportunistic and pathogenic bacteria at their portal of entry.

Physiologically, *Alcaligenes* are known to bear a nitric oxide (NO) reductase gene and reduce NO (32), which was recently reported to up-regulate IgA class-switch recombination (33). These findings suggest that *Alcaligenes* possess unique functions to exclusively coexist in the PPs and to create an optimal environment for their cohabitation through the induction and regulation of mucosal Abs. In general, IgM^+ B cells, a major source for µ to α class switching, are a dominant B cell fraction in PPs of naïve mice (≈70%) (34). Under the appropriate molecular environment including TGF-β1, CD40L, and IL-4 (4), these B cells undergo class switching to IgA-committed B cells, and thus ≈5% of the total cells in PPs are IgA^+ B cells (34). Because NO has been shown to be an additional key regulatory molecule for TNFα/iNOS-producing DC (tip-DC) mediated IgA class switching (33), it is interesting to postulate that NO reductase produced by tissue-inhabiting *Alcaligenes* may serve as a regulatory molecule for the creation of an optimal and steady rate of IgA^+ B cell generation in the PPs.

Unexpectedly, we also detected *Pseudomonas* spp. (genetically homologous with *Pseudomonas fluorescens*) and *Stenotrophomonas* spp. (closely related to *Stenotrophomonas maltophilia*) within the systemic- (or splenic-) but not PP-DCs of naïve, SPF mice (Fig. S2). These two bacteria are considered to be nosocomial pathogens with low levels of virulence in the natural cohabitation state (35, 36). It has also been reported that they spontaneously emerge in immunocompromised cancer patients in the absence of contamination from their surrounding environment (37, 38). Therefore, our present findings may be of crucial clinical significance for a possible role of the intratissue cohabitation by commensal opportunistic bacteria in systemic lymphoid tissues. This line of investigation is now being intensively studied in our laboratory to further elucidate the significance of commensal microbiota that inhabits both systemic and mucosal lymphoid tissues.

In summary, the present study has indicated a unique aspect of mutualism of indigenous opportunistic bacteria with the host immune system in the GI tract. By cohabiting within the organized lymphoid tissues (e.g., PPs and ILFs), these bacteria affect the development and maturation of the host mucosal immune system. Further, the PP-inhabiting, commensal microbiota are an additional element that contributes to creating and maintaining immunologic homeostasis in the host. The universality for the concept of intratissue habitation of *Alcaligenes* is shared by mice and primates, and perhaps other mammals, because their presence inside PPs was demonstrated in mice, monkeys, and humans.

Materials and Methods

Animals and Human Samples. BALB/c and C57BL/6 mice were obtained from CLEA Japan. CBA/N *xid* and control DBA/2 mice were purchased from Japan SLC. $TCR\beta^{-/-}\ \delta^{-/-}$ mice were obtained from the Jackson Laboratory. $IgA^{-/-}$ mice were originally generated by Dr. Gregory Harriman and were kindly provided by the Baylor College of Medicine. Mice were maintained under SPF conditions at the Institute of Medical Science, University of Tokyo and the Immunobiology Vaccine Center, University of Alabama at Birmingham (UAB). GF mouse experiments were performed at the Yakult Central Institute for Microbiological Research. All experiments were conducted in accordance with the guidelines for the Animal Care and Use Committees of the University of Tokyo and UAB.

Nonhuman primate PPs were obtained from cynomolgus macaques housed in the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation (Tsukuba, Japan). All procedures were conducted in accordance with the guidelines for the Animal Care and Use Committees of the TPRC.

Human PPs were kindly provided by healthy patients without irritable bowel disease who underwent endoscopic biopsy at Osaka University Hospital. All of the subjects provided written informed consent, and the study protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine (approval no. 08243) and Institute of Medical Science, University of Tokyo (IMSUT) (approval no. 20-67-0331).

16S rRNA Analysis. The 16S rRNA gene was amplified by PCR with two universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-GGTTACC-

TTGTTACGACTT-3') ligated into plasmid vector pCR2.1 and transformed into INVαF' competent cells by using a TA Cloning Kit (Invitrogen). Plasmid DNA of randomly selected transformants was prepared by using a TempliPhi DNA Amplification Kit (GE Healthcare) and sequenced by using the primers 27F and 520R (5'-ACCGCGGCTGCTGGC-3'). All sequences were examined by BLAST search to identify the closest relatives. Representative nucleotide sequences obtained in this 16S rRNA gene clone library analysis have been deposited in the International Nucleotide Sequence Database (accession nos. AB453241–AB453250).

Whole-Mount FISH Analysis. To detect the domain *Bacteria* or *Alcaligenes*, oligonucleotide probes were purchased from Invitrogen-Molecular Probes (Table S1). Isolated tissue segments were fixed in 4% paraformaldehyde at 4 °C overnight and washed with PBS. Tissues were hybridized in hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl, 45% (ALBO34a, BPA) or 0% (EUB338) formamide, 0.1% SDS, and 10 μg/mL DNA probe] at 60 °C (ALBO34a, BPA) or 42 °C (EUB338) overnight. After washing twice in washing buffer [0.45 M NaCl, 20 mM Tris-HCl, 45% (ALBO34a, BPA) or 0% (EUB338) formamide, and 0.01% SDS] at 60 °C (ALBO34a, BPA) or 42 °C (EUB338) for 10 min, tissue segments were flushed with PBS. Lectin-labeling experiments were performed Alexa Fluor 633-labeled WGA (Invitrogen-Molecular Probes) and biotinylated UEA1 (Vector Laboratories) followed by Alexa 633-conjugated streptavidin (Molecular Probes) at a concentration of 10 μg/mL for 1 h. After being washed with PBS, the tissue samples were mounted and examined by DM IRE2/TCS SP2 confocal microscopy (Leica Microsystems).

Statistical Analysis. Data were expressed as the mean ± SD or SEM and evaluated by an unpaired Student's *t* test. Significance was defined as $P < 0.01$.

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9 Analysis of Intestinal T Cell Populations and Cytokine Productions

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Analysis of Intestinal T Cell Populations and Cytokine Productions

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♦♦♦♦♦♦ I. INTRODUCTION

Intestinal tissues act as the frontlines of the host defense against large numbers of antigens and microorganisms at the most harsh environment in our body. To achieve immunosurveillance and immunological homeostasis in the gut, intestines establish the unique mucosal immune system tightly regulating a state of opposing but harmonized immune activation and quiescence (Kiyono *et al.*, 2008). Accumulating evidence has revealed that numerous types of immunocompetent cells are involved in the maintenance of an appropriate immunological environment of the mucosal immune system. Although the intestinal immune system shared some common immunological features with the systemic immune system, they also show distinct and unique immunological features (Kunisawa *et al.*, 2008).

Among various immunocompetent cells presented at the intestinal tissues, $CD4^+$ T cells play a key role in the regulation of harmonized mucosal immune responses. Classically, $CD4^+$ T cells are divided into two subsets, namely Th1 and Th2 cells, according to their distinct cytokine production profiles which account for two major functions (e.g. cell-mediated immunity [CMI] and humoral-mediated immunity in host immune responses, respectively) (Mosmann and Coffman, 1989; Street and Mosmann, 1991). It is well established that Th1 cells secrete interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α and function in CMI for protection against intracellular bacteria and viruses. In this regard, it has been shown that $CD8^+$ T cells, through their production of IFN-γ, are closely related to

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and play a central role in their cytotoxic functions (Mosmann and Coffman, 1989; Street and Mosmann, 1991). Furthermore, Th1 cells also provide limited help for B cell responses where IFN-γ supports μ to γ2a switches and IgG2a synthesis in mice (Mosmann and Coffman, 1989; Street and Mosmann, 1991). By contrast, the Th2 cells preferentially secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and provide effective help for B cell responses, in particular for IgG1 (and IgG2b), IgE and IgA antibody synthesis (Coffman *et al.*, 1987; Beagley *et al.*, 1988, 1989; Harriman *et al.*, 1988). Thus, numerous numbers of Th2 cells are observed in the mucosal tissues for the preferential induction and regulation of IgA B cell responses.

In addition to classical Th1/Th2 paradigm, recent studies have discovered novel T cell subsets involving in the pro- and anti-inflammatory responses. One subset is $CD4^+$ T cells producing IL-17 and is known as Th17 cells (Harrington *et al.*, 2005; Littman and Rudensky, 2010; Weaver *et al.*, 2007). Like Th1 and Th2 cells, Th17 cells act as effector cells to exclude pathogens by inflammatory responses. On the other hand, it has been shown that $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (known as regulatory T [Treg] cells) play a critical role in the down-regulation of immune responses by IL-10 production and cell–cell interaction (Hand and Belkaid, 2010; Littman and Rudensky, 2010; Sakaguchi *et al.*, 2008). Another regulatory T cell population is known as Tr1 cells, which also produce IL-10 but lack the expression of Foxp3 (Groux *et al.*, 1997; Asseman and Powrie, 1998). It should be noted that these novel types of T cells are preferentially observed in the mucosal tissues, especially in the intestine. Therefore, it is essential to examine cytokine responses in order to characterize the nature of immune responses induced at different stages of host–pathogen interactions or inflammatory responses in the intestine.

Several important cytokines influence the process of generation and development of these T cell subsets. For example, IL-12 and IL-4 direct $CD4^+$ T cells to

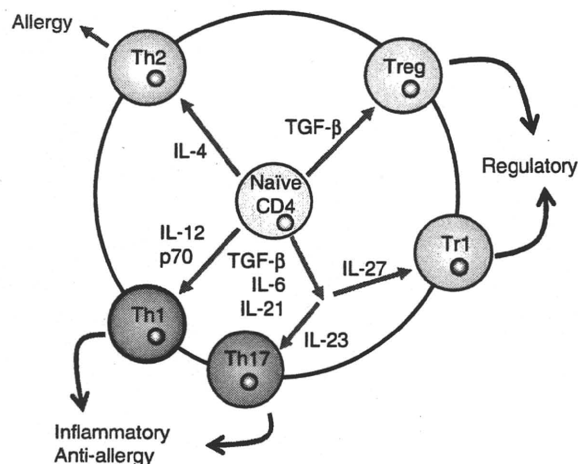

Figure 1. Versatile T cell network in the intestine. Naïve $CD4^+$ T cells activated in the presence of TGF-β differentiate into Treg cells. IL-10-producing Tr1 cells is another type of regulatory T cell induced by TGF-β and IL-6, 21 and 27. On the other hand, IL-23 and IL-12 p70 are involved in the induction of Th17 and Th1 cells, respectively. Th2 cells, a major T cell population in the development of allergic responses, require IL-4.

the differentiation into Th1 and Th2 cells, respectively, while later in development IFN-γ and IL-10 (together with IL-4) can reinforce Th1 or Th2 phenotype expansion (Seder and Paul, 1994). Transforming growth factor (TGF)-β and IL-2 promote the differentiation of Foxp3^{+} Treg cells (Chen *et al.*, 2003). Although TGF-β is also a prerequisite factor for the differentiation of Th17 and Tr1 cells, IL-6 and IL-23 are additionally required for Th17 cell development (Bettelli *et al.*, 2006; Zhou *et al.*, 2007), whereas IL-6 and IL-27 enhance the Tr1 cell differentiation (Stumhofer *et al.*, 2007) (Figure 1).

♦♦♦♦♦♦ II. CELL ISOLATION FROM INTESTINAL TISSUES

A. Background

Intestinal tissues are generally and functionally divided into two sites. One is organized lymphoid organs and acts as the inductive site for the initiation of antigen-specific immune responses. Peyer's patches (PPs) are representative lymphoid organs in the intestine and known as a member of gut-associated lymphoid tissues (GALTs) (Kunisawa *et al.*, 2008) (Figure 2A). PPs show the features of

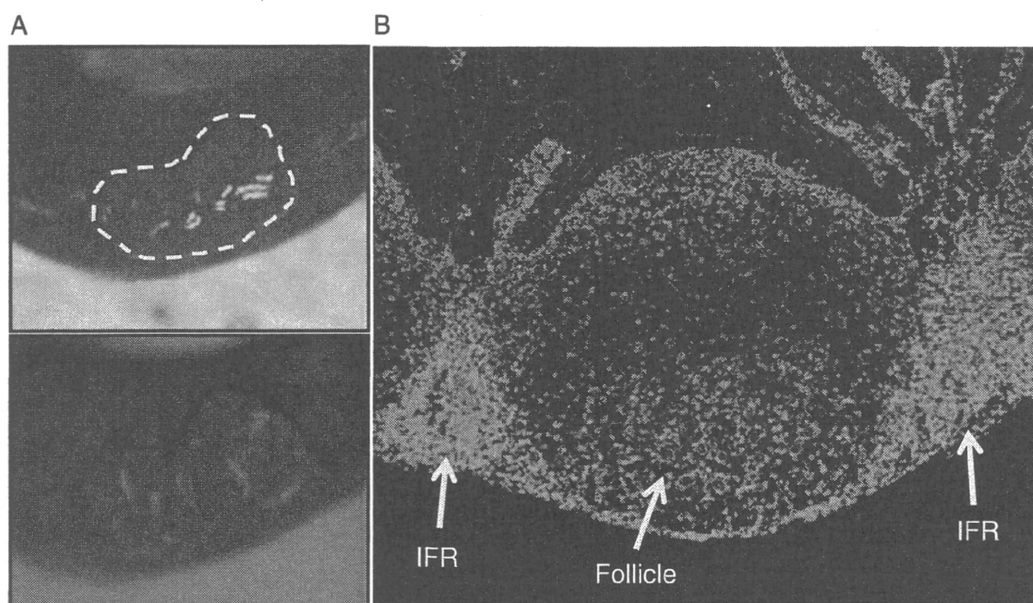

Figure 2. Macroscopic and histological views of Peyer's patches. (A) Mice were adoptively transferred with green fluorescent dye (carboxyfluorescein succinimidyl ester)-labelled naïve T cells. Sixteen hours later, small intestine was observed by conventional (upper) and fluorescent (bottom) stereomicroscopy. Yellow line in upper picture indicates the place of Peyer's patch. (B) Immunohistochemical data on Peyer's patch are shown. CD4^{+} T cells (green) are present mainly in the intrafollicular regions (IFRs) and follicle. (See color plate section).

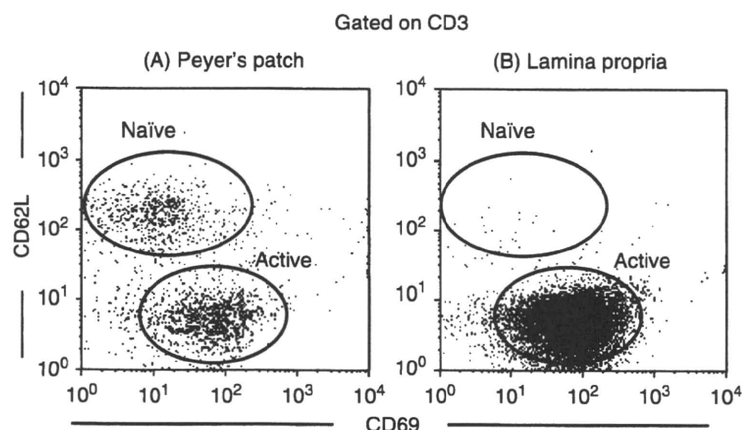

Figure 3. Immunological phenotypes of intestinal T cells. Cells were isolated from the Peyer's patches (A) and intestinal lamina propria (B), and stained with fluorescent-labelled antibodies for CD3, CD62L and CD69. The figures show the naïve ($CD62L^{hi}$ $CD69^-$) and activated ($CD62L^-$ $CD69^+$) cells in $CD3^+$ T cells.

secondary lymphoid organs and thus contain naïve T cells, especially at the interfollicular region (IFR: Figures 2B and 3A). In the IFR, naïve T cells recognize antigen presented by dendritic cells and subsequently differentiate into activated Th1- or Th2-type T cells in the follicle (Figures 2B and 3A). The other part is lamina propria region containing various types of T cells such as Th1, Th2, Th17, Tr1 and Treg cells for the execution of different effector functions including active and quiescent immune responses and thus known as the effector site. Under the epithelium, T cells exist diffusely with IgA^+ plasma cells (Figure 4) and show activated phenotype mainly (Figure 3B).

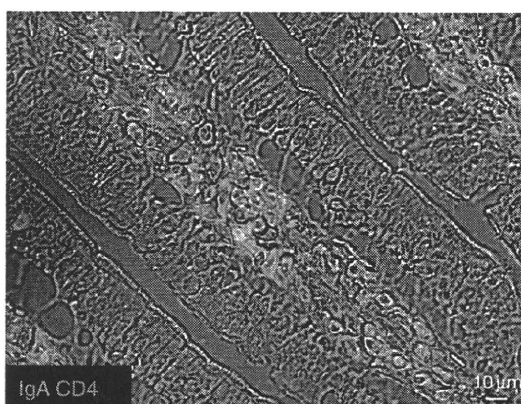

Figure 4. Distribution of immunocompetent cells in the intestinal lamina propria. Immunohistochemical data on intestinal lamina propria are shown. $CD4^+$ T cells (red) and IgA^+ plasma cells (green) are diffusely present in the lamina propria region of small intestine. (See color plate section).

B. Isolation Lymphocytes from the Peyer's Patches (PPs)

1. Isolate the intestines and remove the PPs carefully using scissors.
2. Cut into small pieces as possible by scissors.
3. Incubate the pieces in 15 ml of pre-warmed RPMI1640 medium containing 2% foetal calf serum (FCS) plus 0.5 mg/ml of collagenase (available from many companies, but activity is different among companies and their lot. Therefore, it is necessary to check the activity and determine the optimal concentration). Stir the intestine for 20 min at 37°C.
4. Collect the supernatants in a fresh 50-ml tube and centrifuge for 5 min at 500 × *g* at 4°C. Suspend pellet with RPMI1640 containing 2% FCS.
5. Repeat twice steps 3 and 4.
6. Combine all cells and pass the cells through a 80-µm cell strainer. Centrifuge for 5 min at 500 × *g* at 4°C.
7. Suspend the cells with appropriate solution for further analysis.

Analysis of Intestinal T Cell Populations and Cytokine Productions

C. Isolation Lymphocytes from the Intestinal Lamina Propria

1. Isolate the intestines and remove the PPs.
2. Open the intestine longitudinally, and wash it with ice-cold RPMI1640 medium (no FCS). Place the intestine in ice-cold RPMI1640 medium containing 2% FCS.
3. Cut the intestine into 2–3 cm pieces by scissors and incubate the pieces in 25 ml of pre-warmed (37°C) RPMI1640 medium containing 2% FCS and 0.5 mM ethylenediaminetetraacetic acid (EDTA). Stir the intestine in conical flask for 20 min at 37°C.
4. Remove the solution by passing the intestine through stainless mesh (e.g. a tea strainer). Put the intestine in a 50-ml tube containing 20 ml of plain RPMI1640 medium and shake them vigorously (~15 s).
5. Repeat step 4 once again.
6. Incubate the pieces in 25 ml of pre-warmed RPMI1640 medium containing 2% FCS. Stir the intestine in conical flask for 20 min at 37°C.
7. Repeat step 4 twice.
8. Cut into small pieces by scissors.
9. Incubate the pieces in 15 ml of pre-warmed (37°C) RPMI1640 medium containing 2% FCS plus 0.5 (small intestine) or 1.0 (large intestine) mg/ml of collagenase (concentration is dependent on the lot). Stir the intestinal pieces for 20 min at 37°C.
10. Collect the cell suspensions in a fresh 50-ml conical tube and centrifuge for 5 min at 500 × *g* at 4°C. Suspend pellet with RPMI1640 containing 2% FCS and pass the cell suspensions through a 100-µm cell strainer.
11. Repeat steps 9 and 10 twice.
12. Combine all cells and pass them through a 80-µm cell strainer. Centrifuge for 5 min at 500 × *g* at 4°C.
13. Suspend the pellet with 40% Percoll solution and overlay the cell suspension on 75% Percoll solution (Figure 5). Centrifuge for 20 min at 900 × *g* at 20°C without brakes.

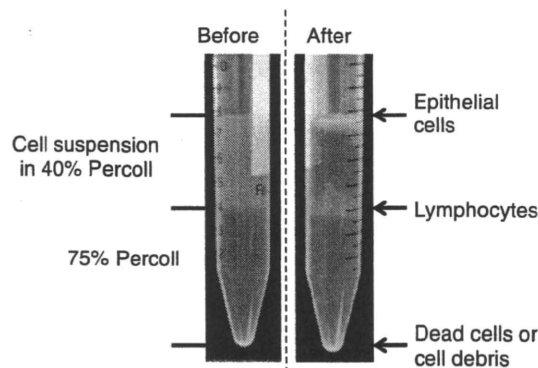

Figure 5. Cell purification using Percoll gradient centrifugation. To remove the epithelial cells, cell debris and dead cells, Percoll gradient centrifugation was performed. Initially, cells were suspended in the 40% of Percoll solution and put on the 75% of Percoll solution (before). After the centrifugation, lymphocytes were observed at the interphase between 40 and 75% Percoll solution. Epithelial cells and dead cells plus cell debris are observed at the top of layer and the bottom of the tube, respectively.

14. Collect cells at the interphase between 40 and 75% Percoll solutions (some epithelial cells are observed at the top of layer and debris and dead cells are at the bottom of the tube) (Figure 5).
15. Wash cells with 30 ml of RPMI1640 plus 2% FCS and centrifuge the cell suspension for 5 min at 500×g at 4°C.
16. Suspend the cells with appropriate solution and use for analysis.

♦♦♦♦♦♦ III. MEASURING CYTOKINE PRODUCTION FROM INTESTINAL T CELLS

A. Background

Cytokines are important biological molecules regulating distinct functions of different immunocompetent cells. As indicated above, T cells can be divided into several populations by the cytokine productions. Various techniques for the detection of cytokine production and/or expression have proven to be valuable for studies of T cell-mediated immune responses and examine the outcome of vaccine- and immune therapy-induced responses. We describe here three of these commonly used techniques to detect murine cytokines productions at protein levels and show some representative data on various cytokine productions by intestinal T cells. First, enzyme-linked immunosorbent assay (ELISA) assay can enumerate the amounts of produced cytokines from T cells. Second, ELISPOT assay is used to quantify the numbers of T cells producing particular cytokines (Czerkinsky *et al.*, 1988). Third, Intracellular cytokine staining assay can determine the T cell subsets and frequencies producing the specific cytokines when the cell were simultaneously stained with subset-specific markers.

B. Cytokine-Specific ELISA

For the analysis of murine cytokines, various kinds of ELISA kits are currently available from many companies. In addition, wide-ranging cytokine assays such as cytokine bead array (BD Biosciences, San Jose, CA) and Bio-plex system (Bio-rad, Richmond, CA) are currently available. Therefore, we summarize here the basic protocol by cytokine-specific ELISA system. We also show the example of cytokine production of small intestinal $CD4^+$ T cells (Figure 6).

1. Dilute the capture antibody in phosphate-buffered saline (PBS) and add 100 μl to the wells of 96-well microtitre plates (e.g. Immulon [Thermo Fisher Scientific, Rochester, NY]). Incubate the plates overnight at 4°C.
2. Remove the antibody solution from wells and block the coated antibody with PBS containing 1% BSA for 1 h at room temperature.
3. Wash the plates three times with PBS.
4. Prepare the standard curves using recombinant cytokines (e.g. two-fold serial dilutions in PBS containing 0.5% Tween 20 [PBS-T]).
5. To obtain the T cell culture supernatant, $2–10 \times 10^4$ purified T cells were stimulated with immobilized anti-CD3 antibody (clone: 145-2C11; 1–5 μg/ml in PBS) plus 1 μg/ml of anti-CD28 antibody (clone: 37.51) for 72–96 h at 37°C. Alternatively, antigen-primed T cells ($2–10 \times 10^4$ cells) are stimulated with appropriate antigen plus antigen-presenting cells (e.g. irradiated splenocytes) for 96 h at 37°C.
6. Add 100 μl of cytokine standards or appropriately diluted T cell culture supernatants and incubate the plates overnight at 4°C.
7. Wash the plates four times with PBS-T.
8. Add 100 μl of appropriate biotinylated capture antibody diluted in PBS-T with 1% BSA. Incubate the plates overnight at 4°C.
9. Repeat step 7.
10. Add 100 μl of peroxidase-labelled anti-biotin antibody and incubate the plates for 1 h at room temperature.

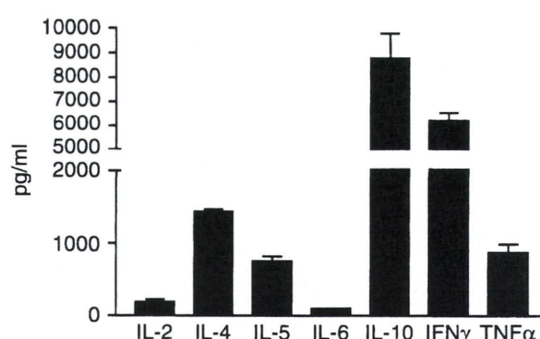

Figure 6. Cytokine productions by activated intestinal $CD4^+$ T cells. Lymphocytes were isolated from the small intestine and applied to the FACS cell sorting to purify the $CD4^+$ T cells. For the stimulation of T cells, 2×10^4 purified $CD4^+$ T cells were cultured with immobilized anti-CD3 antibody plus 1 μg/ml of anti-CD28 antibody for 96 h at 37°C. Cytokine production in the culture supernatant was determined by cytokine-specific ELISA.

11. Repeat step 7.
12. Develop the colour with appropriate chromogenic substrates (e.g. TMB microwell peroxidase substrate system [KPL, Gaithersburg, MD]) and read the absorbance.
13. Calculate the concentrations of samples by reference to the liner portion of the standard curve.

C. Cytokine-Specific ELISPOT

Like cytokine ELISA assay, several ELISPOT kits are commercially available. Thus, we summarize here a basic protocol of cytokine ELISPOT assay.

1. Dilute the capture antibody in PBS and add 100 µl to the wells of 96-well nitrocellulose-backed microtitre plate (e.g. Millititer-HA [Millipore, Billerica, MA]). Place the plates in a humidified chamber or carefully wrap the plate in saran wrap and incubate overnight at 4°C.
2. Remove the antibody solution from wells and block the immobilized antibody with culture medium (e.g. RPMI1640 medium containing 10% FCS) for 1 h at 37°C.
3. Rinse the plate three times with PBS.
4. Prepare the five-fold dilutions of purified T cells in culture medium starting at $1–10×10^6$ cells/ml. Immediately add 100 µl of cells and incubate them for 12–16 h at 37°C. The time required for T cell purification significantly reduces the numbers of detectable cytokine-producing cells. Therefore, it is important to prepare the cells in a prompt manner.

   For the assessment of cytokine productions by antigen-specific T cells, purified T cells should be re-stimulated with the same antigens in the presence of irradiated antigen-presenting cells. Between 1 and 6 days after antigen stimulation, T cells are harvested and immediately added to the capture antibody-coated plates as described above.
5. Wash the plates three times with PBS followed by three times washes with PBS-T.
6. Add 100 µl of appropriate biotinylated capture antibody diluted in PBS-T with 1% BSA. Incubate the plates overnight at 4°C.
7. Wash the plates six times with PBS-T.
8. Add 100 µl of peroxidase-labelled anti-biotin antibody and incubate the plates for 1 h at room temperature.
9. Wash the plates four times with PBS.
10. Develop the colour with appropriate chromogenic substrates (e.g. AEC [BD Biosciences]) and count red spots by stereomicroscope or automated ELISPOT readers (e.g. KS ELISPOT [Carl Zeiss, Oberkochen, Germany]).

D. Intracellular Cytokine Staining

Using intracellular cytokine staining method, the frequency of cytokine-producing cells and their phenotypes can be determined by flow cytometer. By using subset-

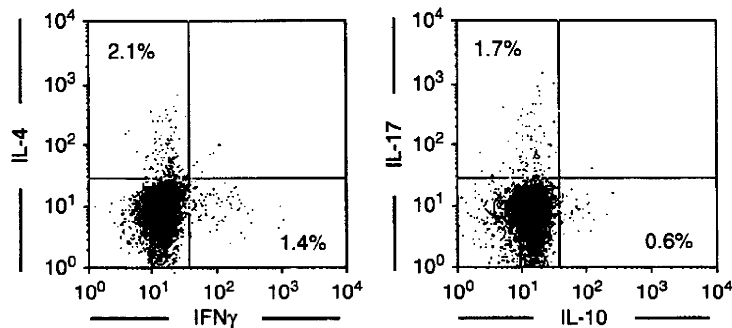

Figure 7. Intracellular cytokine staining of small intestinal T cells. Lymphocytes were isolated from the small intestine and cultured with 50 ng/ml PMA, 5 μM calcium ionophore A23187 and golgistop (BD Biosciences) for 4 h at 37°C. Cells were stained with anti-CD3 antibody followed by the fixation and permeabilization of cell membrane by Cytofix/Cytoperm kit (BD Biosciences). The permeable cells were further stained with antibodies specific for each cytokine and analysed by flow cytometry.

specific antibody, we do not need to purify the T cells. As example, we show here the data on cytokine-producing $CD4^+$ T cells isolated from small intestines (Figure 7).

1. Incubate lymphocytes in culture medium with 50 ng/ml PMA, 5 μM calcium ionophore A23187 and golgistop (BD Biosciences) for 4 h at 37°C.
2. Harvest the cells and stain cells with a corresponding cocktail of fluorescently labelled antibodies for 30 min at 4°C.
3. Wash the cells twice with PBS plus 2% FCS (PBS-F).
4. Fix the stained cells with 250 μl of Cytofix/Cytoperm solution (BD Biosciences) or 2% paraformaldehyde for 20 min at 4°C.
5. Wash cells twice with 1 ml of Perm/Wash buffer (BD Biosciences).
6. Incubate cells with fluorescently labelled cytokine-specific antibodies for 20 min at 4°C.
7. Repeat step 5 and suspend cells with PBS-T.
8. Analyse with Flow cytometer.

♦♦♦♦♦♦ IV. CONCLUSION

In this chapter, we have described the protocol for the analysis of T cell population in the intestine and their cytokine productions. For the cytokine production assay, we show three different methods: ELISA, ELISPOT and intracellular cytokine staining. These three assay systems allow the detection of different stages of cytokine production. Although each assay has unique advantages for the detection of T cell cytokines, the use of individual assays in a separate manner may often not

be sufficient for a thorough and accurate determination of the T cell cytokine profiles. Additionally, recent advances in the imaging technologies allow us to observe the cytokine-producing cells *in vivo* (Kamanaka *et al.*, 2006). Thus, combining traditional technologies with the modern and novel technologies will lead to the better understanding of T cell responses in the intestine.

Acknowledgements

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