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〈雑誌〉

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Ⅴ．　研究成果の刊行物・別冊
（主なもの）

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.

***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

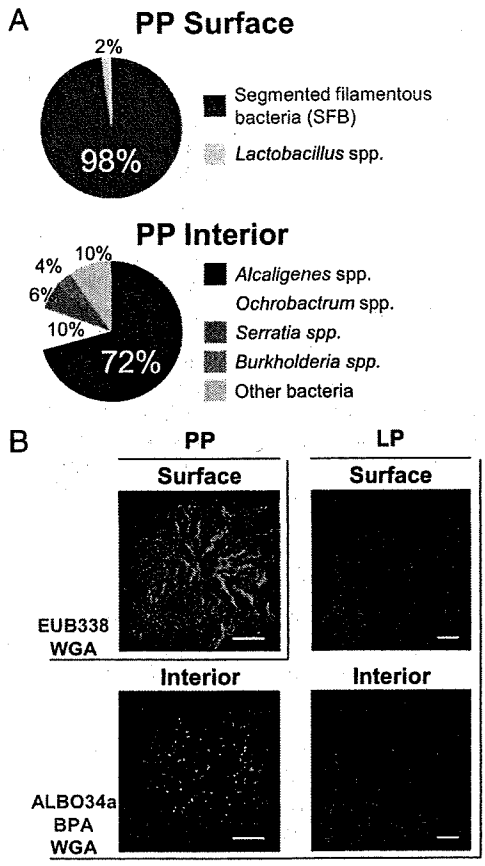

Fig. 1. Microbial distribution in the GI immune compartment. (*A*) Microbial composition at the surface and on the interior of PPs was examined by 16S rRNA gene clone library analysis. (*B*) The presence of *Alcaligenes* was visually analyzed by whole-mount FISH at the surface and on the interior of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

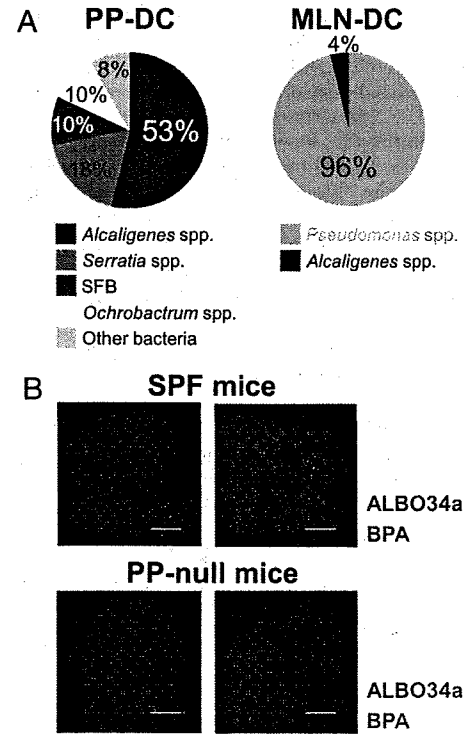

Fig. 2. PP-MLN migration axis for *Alcaligenes*-ingested GI tract DCs. (*A*) $CD11c^+$ DCs were isolated from the PPs and MLNs. Bacterial composition was determined by 16S rRNA gene clone library analysis. (*B*) Whole-mount FISH was performed to detect *Alcaligenes* (yellow) in the MLNs of PP-intact 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*-

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).

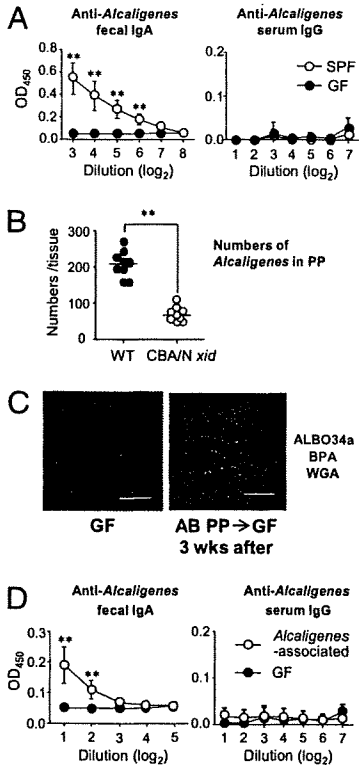

Fig. 3. Preferential induction of *Alcaligenes*-specific mucosal Ab responses in the PPs. (*A*) *Alcaligenes*-specific fecal IgA and serum IgG Ab responses were determined by ELISA. Data are means ± SD (n = 4). (*B*) The numbers of *Alcaligenes* inside PPs were counted in 10 randomly chosen PPs of CBA/N *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 monoassociated 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

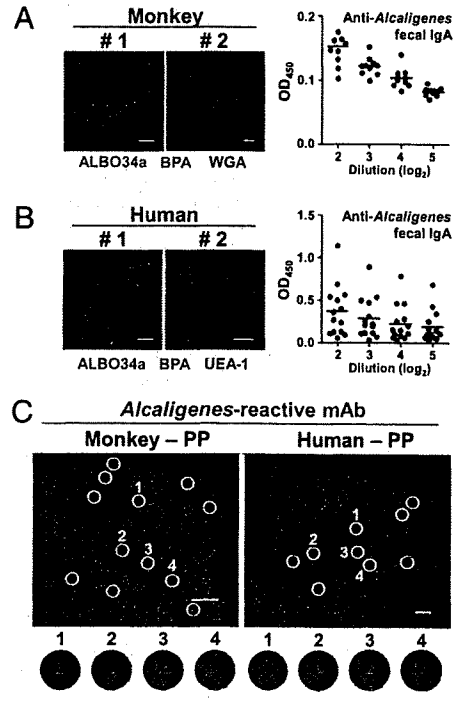

Fig. 4. Intratissue habitation of *Alcaligenes* inside nonhuman primate and human PPs. (*A* and *B*) *Alcaligenes* were detected on the interior of monkey and human PPs by whole-mount FISH (*Left*). *Alcaligenes*-specific fecal IgA Ab responses in monkeys and human were examined by ELISA [*Right*; n = 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 × 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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Supporting Information

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SI Materials and Methods

Preparation of PP-Null Mice and Antibiotic Treatment. Peyer's patch (PP)-null mice were generated by treating pregnant mice with an anti-IL-7Rα mAb as described previously (1). The absence of PPs was confirmed histologically.

Antibiotic-treated mice were provided with water containing ampicillin (1 g/L), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L) for 1 month.

Bacteria. *Alcaligenes faecalis* subsp. *faecalis* [National Institute of Technology and Evaluation Biological Resource Center (NBRC) 13111^T] was purchased from the NBRC and grown in Tryptic Soy Broth (BD Diagnostics) at 37 °C. GFP-*Alcaligenes* were generated by inserting the *gfp* gene into the *Eco*RI-*Hin*dIII site of plasmid pMMB503EH conferring streptomycin resistance (2), giving rise to the isopropyl β-D-thiogalactoside (IPTG)-inducible pMMB503-GFP. This plasmid was then transformed into NBRC 13111^T by conjugation via *Escherichia coli* S17-1. GFP-*Alcaligenes* were grown at 37 °C in Tryptic Soy Broth containing streptomycin (200 μg/mL) and IPTG (1 mM). Kusabira Orange–*E. coli* DH5α was generated by the transformation of plasmid pKO1-S1 (MBL) into competent *E. coli* DH5α (TOYOBO) and grown in Luria-Bertani broth containing ampicillin (100 μg/mL).

Sample Preparation for 16S rRNA Analysis. PPs were isolated from the small intestines of specific-pathogen-free (SPF) and germ-free (GF) mice as described previously (3). After feces were removed, the tissues were washed vigorously five times with PBS, and washing fluids were collected by centrifugation at 20,000 × *g* for 10 min (and served as the *PP surface sample*). The PP segments were vigorously agitated in EDTA solution (0.5 mM EDTA in RPMI 1640) to remove epithelial cells. After being washed vigorously, they were gently agitated in collagenase type IV solution (0.5 mg/mL collagenase IV in RPMI 1640), and a single-cell suspension was prepared. The supernatant of the collagenase-treated fluid was centrifuged at 1,900 × *g* for 10 min to remove host mononuclear cells and then centrifuged at 20,000 × *g* for 10 min (and used as *PP interior samples*). The cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech) and subjected to Magnetic Cell Separation System (MACS) columns (Miltenyi Biotech). Purified cells were lysed with 0.2% (wt/vol) deoxycholate by vortexing for 1 min, and the bacteria within PP $CD11c^+$ cells were collected by centrifugation at 20,000 × *g* for 10 min (and served as *PP $CD11c^+$ cell samples*). Spleen and mesenteric lymph nodes (MLNs) were dissected, and single-cell suspensions were prepared by treatment with collagenase type IV solution. $CD11c^+$ cells were purified by MACS (Miltenyi Biotech), and bacteria within splenic and MLN $CD11c^+$ cells were collected after deoxycholate treatment as described above (and analyzed as *splenic $CD11c^+$* and *MLN $CD11c^+$ cell samples*, respectively).

Generation of *Alcaligenes*-Specific mAbs. Mononuclear cells isolated from murine PPs were randomly fused with P3 × 63-AG8.653 myeloma cells (American Type Culture Collection, CRL-1580) in the presence of 50% (wt/vol) polyethylene glycol 1500 (Roche Diagnostics). Established hybridomas were screened for the production of Abs highly reactive or nonreactive to *Alcaligenes* NBRC 13111^T by ELISA. These mAbs were then purified by using KAPTIV-AE (Tecnogen). The lines of *Alcaligenes*-specific mAb #11E-8C-7A (IgM isotype) and nonspecific mAb #12A-5G-4F (IgM isotype) were used for the study.

Immunohistochemistry. Tissue segments containing PPs were dissected from monkey and human small intestine and fixed in 4% paraformaldehyde at 4 °C overnight. After being washed with PBS and sucrose-containing buffer, they were frozen in Tissue-Tek O.C. T. Compound (Sakura Finetek). Frozen tissue sections were resected and reacted with *Alcaligenes*-reactive #11E-8C-7A or -nonreactive mAbs #12A-5G-4F at 4 °C overnight. They were blocked with rat IgG Abs and stained with phycoerythrin-conjugated anti-mouse IgM Ab (BD PharMingen). After DAPI staining, the tissue samples were mounted and examined by DM IRE2/TCS SP2 confocal microscopy (Leica Microsystems).

ELISA. To examine Ab levels, an ELISA was performed as described previously (4). Briefly, microtiter plates were coated with 100 μL of 1 mg/mL lyophilized NBRC 13111^T or 5 μg/mL goat anti-mouse Ig (Southern Biotech). Wells were blocked with blocking buffer [1% BSA in PBST (0.05% Tween-20 in PBS)]. Serum and fecal extracts were added and incubated. They were incubated with HRP-conjugated anti-mouse IgA or IgG Abs (Southern Biotech). Before each incubation process, the plates were washed with PBST buffer. Finally, they were reacted with 3,3′,5,5′-tetramethylbenzidine for exactly 5 min. The absorbance was read at a wavelength of 450 nm.

ELISPOT Assay. Mononuclear cells from PPs and lamina propria (LP) were isolated from the small intestines of SPF and GF mice as described previously (3). To assess the numbers of Ab-forming cells (AFCs), an enzyme-linked immunospot (ELISPOT) assay was performed as described previously (4). Briefly, nitrocellulose plates were coated with 100 μL of 1 mg/mL lyophilized NBRC 13111^T or 5 μg/mL goat anti-mouse Ig (Southern Biotech). They were blocked with RPMI 1640 containing 10% FCS. The lymphoid cell suspensions at various dilutions were added and incubated. HRP-conjugated anti-mouse IgA or IgG Abs were added and finally reacted with 3-amino-9-ethylcarbazole dissolved in hydrogen peroxide-containing buffer (Moss). The reaction was stopped, and AFCs were counted with the aid of a stereomicroscope (4).

Flow Cytometric Analysis. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences) as described previously for the analysis of antigen-specific IgA^+ B cells (3). In brief, cells were first preincubated with an anti-FcR mAb (2.4G2; BD PharMingen) and then stained with appropriate fluorescence-conjugated Abs specific for IgA (Southern Biotech) and B220 (BD PharMingen). Finally, cells were reacted with the bacteria expressing fluorescent proteins. To examine cross-reactivity, PP cells were first incubated with the fluorescent protein-expressing bacteria and biotin-conjugated anti-mouse IgA mAb (Southern Biotech) and then stained with APC-conjugated streptavidin (BD PharMingen). Data were analyzed by CellQuest software (BD Biosciences).

Cytokine Assay. PP cells (1×10^5 cells) or purified PP-dendritic cells (PP-DCs) (5×10^4 cells) from GF or SPF mice were cultured with live or heat-killed (65 °C, 30 min) NBRC 13111^T (5×10^6 cells) in complete medium (RPMI 1640, 10% heat-inactivated FCS, 50 μM 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL gentamicin) at 37 °C in a humidified atmosphere of 5% CO_2. After 2 days, culture supernatants were collected for measurement of IL-6 production by ELISA (R&D Systems).

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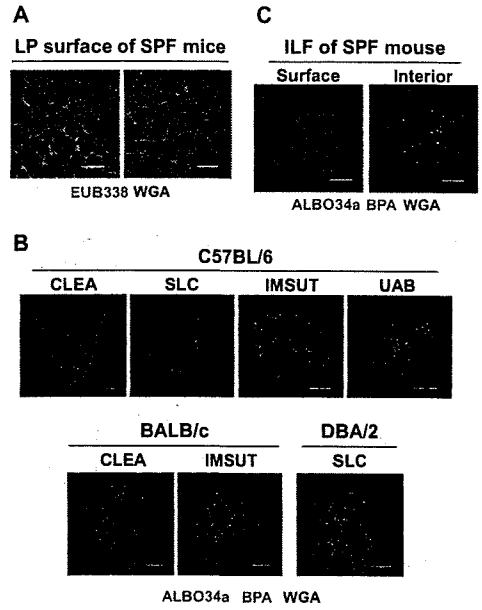


Fig. S1. *Alcaligenes* universally inhabit PPs and isolated lymphoid follicles (ILFs) of the small intestine. (*A*) EUB338-positive bacteria, mostly segmented filamentous bacteria, were detected all over the surface area of LP. Data are representative of three independent experiments. (Scale bars, 150 μm.) (*B*) Presence of ALBO34a and BPA double-positive *Alcaligenes* was confirmed inside PPs of C57BL/6, BALB/c, and DBA/2 mice maintained in different SPF-conditioned facilities of CLEA Japan (CLEA), Japan SLC (SLC), Institute of Medical Science, University of Tokyo (IMSUT), and the Immunobiology Vaccine Center, University of Alabama at Birmingham (UAB). Data are representative of three independent experiments. (Scale bars, 100 μm.) (*C*) *Alcaligenes* were detected on the interior of small intestinal ILFs. Data are representative of three independent experiments. (Scale bars, 50 μm.)

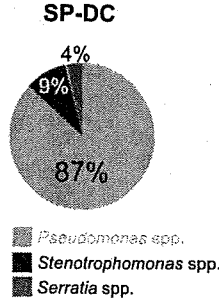


Fig. S2. Bacterial composition inside murine splenic DCs (SP-DC). $CD11c^+$ DCs were isolated from spleen of SPF mice. Bacterial composition was determined by 16S rRNA gene clone library analysis.

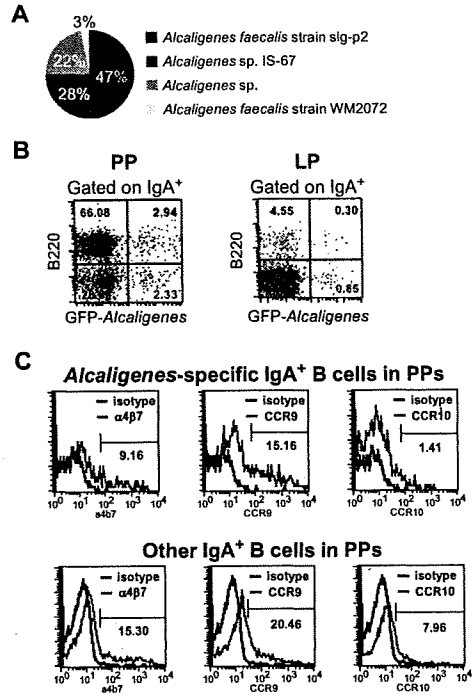


Fig. S3. Preferential induction of *Alcaligenes*-specific IgA-positive B cells in the PPs. (*A*) Classification of the *Alcaligenes* species inside murine PPs. 16S rRNA gene sequences were examined to classify *Alcaligenes* spp. in the murine PPs. (*B*) Flow cytometric data show the presence of GFP-*Alcaligenes* specific IgA$^+$ B220^{high} cells (B cells) and IgA$^+$ B220^{low} cells (plasmablasts/plasma cells) isolated from PPs and LP of SPF mice. Data are representative of three independent experiments. (*C*) Low expression of gut-imprinting receptors by *Alcaligenes*-specific IgA-positive B cells in PPs. Expression levels of gut-homing receptors, α4β7, CCR9, and CCR10, were examined by flow cytometric analysis. Data are representative of three independent experiments.

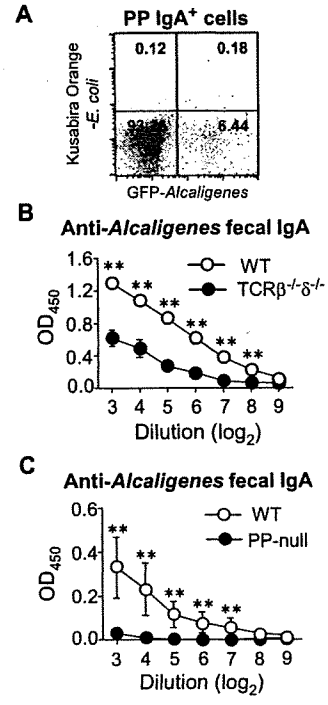


Fig. S4. Reduction or lack of *Alcaligenes*-specific mucosal IgA responses in the T cell-deficient or PP-null mice. (*A*) The cross-reactivity of *Alcaligenes*-specific PP IgA$^+$ B cells with other bacteria was examined. PP IgA$^+$ B cells were isolated and incubated with GFP-*Alcaligenes* and Kusabira Orange-*E. coli* and then subjected to FACS analysis. Data are representative of three independent experiments. (*B*) *Alcaligenes*-specific fecal IgA Ab responses in TCRβ$^{-/-}$ δ$^{-/-}$ mice (n = 8) and WT mice (n = 11) were measured by ELISA. Data are expressed as means ± SEM. $^{**}P < 0.01$. (*C*) *Alcaligenes*-specific fecal IgA Ab responses in PP-null mice were examined by ELISA. Data are means ± SD (n = 12). $^{**}P < 0.01$.

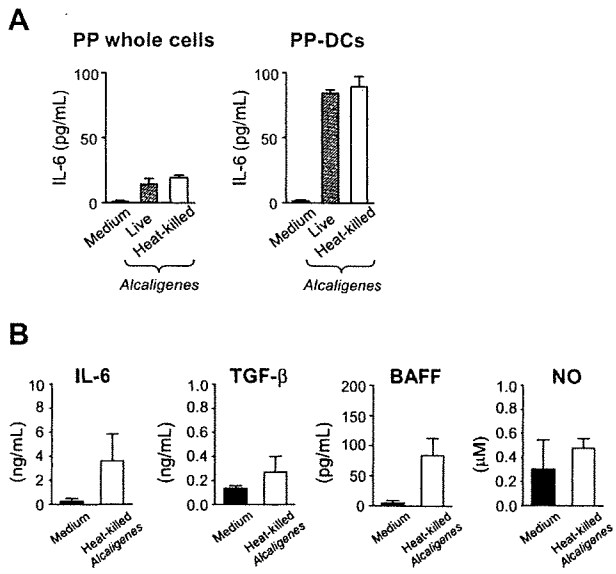


Fig. S5. IgA-enhancing cytokine production by PP-DCs stimulated with *Alcaligenes*. (*A*) Mononuclear cells (1×10^5 cells) or DCs (PP-DCs, 5×10^4 cells) were isolated from PPs of GF mice and cultured with live or heat-killed *Alcaligenes* (5×10^6 cells) for 48 h. Levels of IL-6 production in the culture supernatants were assessed by ELISA. Data are the mean ± SD ($n = 3$), and experiments were repeated at least twice. (*B*) PP-DCs [IL-6, 1×10^5 cells; TGF-β, 1×10^5 cells; B-cell-activating factor belonging to the TNF family (BAFF), 1×10^6 cells; and NO, 5×10^5 cells] were isolated from SPF mice and cultured with heat-killed *Alcaligenes* (IL-6, 1×10^7 cells; TGF-β, 1×10^6 cells; BAFF, 1×10^7 cells; and NO, 5×10^6 cells) for 48 h. Levels of each cytokine production in the culture supernatants were assessed by ELISA. Data are the mean ± SD ($n = 3$) and are representative of three independent experiments.

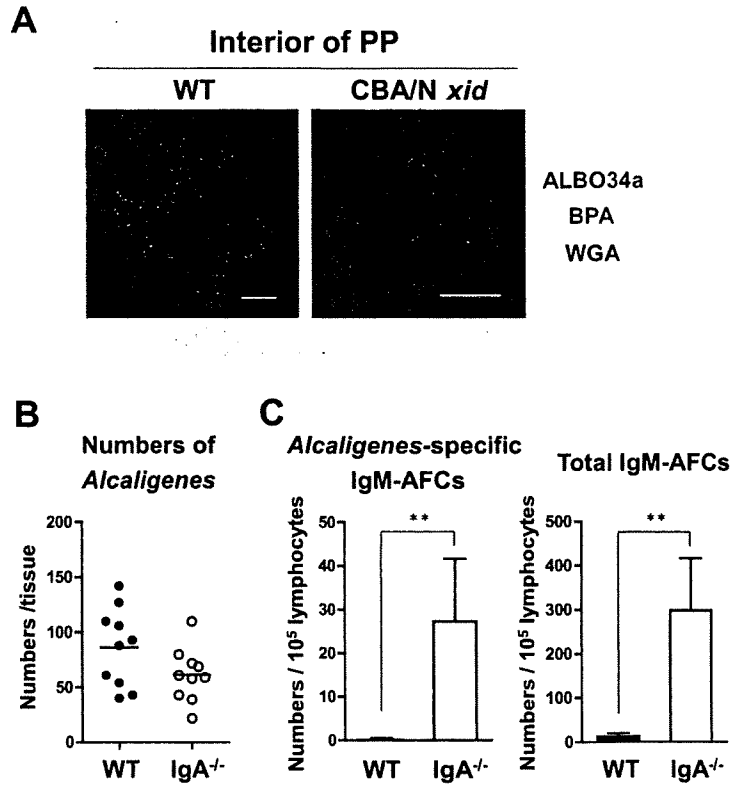


Fig. S6. Mucosal Abs play a critical role in the intratissue cohabitation of *Alcaligenes* in PPs. (*A*) Diminishment of *Alcaligenes* on the interior of PPs in the B cell defect mice. Whole-mount FISH analysis using ALBO34a, BPA, and WGA was performed to detect *Alcaligenes* inside PPs of CBA/N *xid* and WT mice. Data are representative of three independent experiments. (Scale bars, 100 μm.) (*B*) Numbers of intratissue *Alcaligenes* were reduced in PPs of IgA-deficient mice. ALBO34a and BPA double-positive signals detected inside PPs were counted as the numbers of *Alcaligenes* in 10 randomly chosen PPs of IgA-deficient mice. Data are representative of three independent experiments. Horizontal bar indicates the mean. $P > 0.05$. (*C*) Numbers of IgM-AFCs were increased in PPs of IgA-deficient mice. *Alcaligenes*-specific and total IgM-AFCs in WT and IgA-deficient mice were enumerated by ELISPOT assay. Data are expressed as means ± SD (n = 12, respectively). $^{**}P < 0.01$.

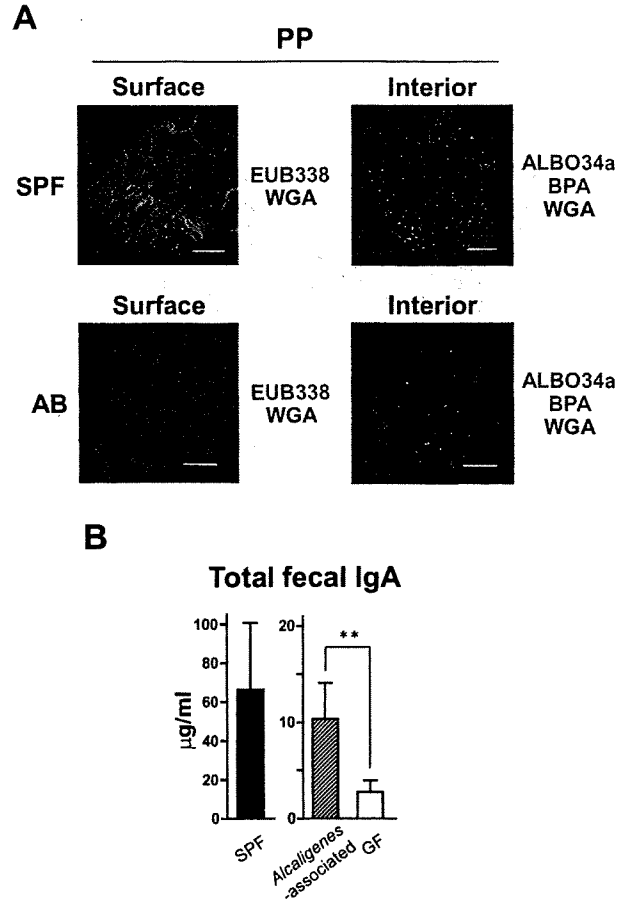


Fig. S7. The oral transfer of intratissue *Alcaligenes* induced total mucosal IgA responses in *Alcaligenes*-associated, ex-GF mice. (*A*) Bacterial distribution at the surface and on the interior of PPs of antibiotic-treated, SPF mice. AB, antibiotic-treated mice. Data are representative of three independent experiments. (Scale bars, 100 μm.) (*B*) ELISA was performed to quantify total IgA in the feces of SPF, *Alcaligenes*-associated ex-GF, and GF mice. Data are expressed as means ± SD ($n = 6$). $^{**}P < 0.01$.

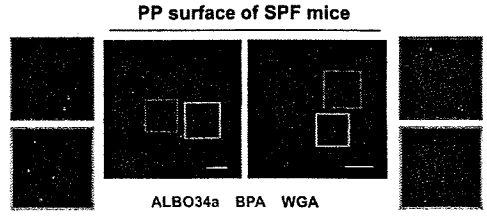
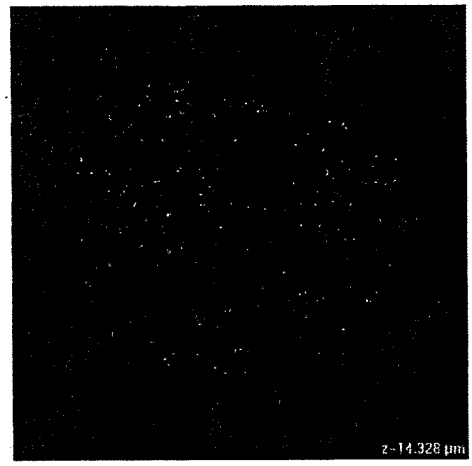


Fig. S8. Presence of *Alcaligenes* at the surface of PPs in the small intestine. ALBO34a and BPA double-positive *Alcaligenes* were detected at the surface of small intestinal PPs. Data are representative of three independent experiments. (Scale bars, 100 μm.)

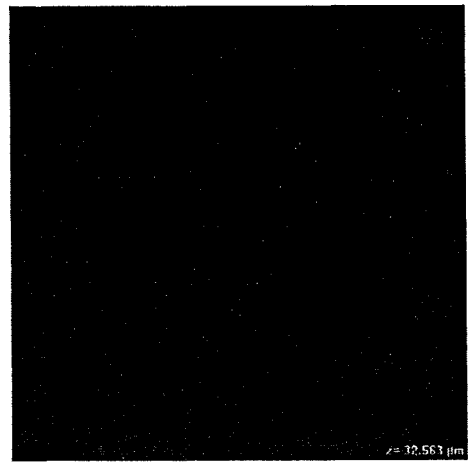
Table S1. Oligonucleotide probes used in the FISH study

Probe name	Specificity	Targeted site(rRNA positions)	5′-label
EUB338	Bacteria	16S (338–335)	Alexa488
ALBO34a	*Alcaligenes* spp. *Bordetella* spp.	23S (699–716)	Alexa594
BPA	*Alcaligenes* spp. *Burkholderia* spp. *Comamonas* spp.	16S (1022–1044)	Alexa488



Movie S1. Alcaligenes inhabit Peyer's patches of gastrointestinal tract.

Movie S1



Movie S2. Alcaligenes inhabit isolated lymphoid follicles of gastrointestinal tract.

Movie S2

RANKL Is Necessary and Sufficient to Initiate Development of Antigen-Sampling M Cells in the Intestinal Epithelium[1]

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Microfold cells (M cells) are specialized epithelial cells situated over Peyer's patches (PP) and other organized mucosal lymphoid tissues that transport commensal bacteria and other particulate Ags into intraepithelial pockets accessed by APCs. The TNF superfamily member receptor activator of NF-κB ligand (RANKL) is selectively expressed by subepithelial stromal cells in PP domes. We found that RANKL null mice have <2% of wild-type levels of PP M cells and markedly diminished uptake of 200 nm diameter fluorescent beads. Ab-mediated neutralization of RANKL in adult wild-type mice also eliminated most PP M cells. The M cell deficit in RANKL null mice was corrected by systemic administration of exogenous RANKL. Treatment with RANKL also induced the differentiation of villous M cells on all small intestinal villi with the capacity for avid uptake of *Salmonella* and *Yersinia* organisms and fluorescent beads. The RANK receptor for RANKL is expressed by epithelial cells throughout the small intestine. We conclude that availability of RANKL is the critical factor controlling the differentiation of M cells from RANK-expressing intestinal epithelial precursor cells. *The Journal of Immunology,* 2009, 183: 5738–5747.

The organized lymphoid tissues of the intestine are inductive sites for both the generation of secretory IgA and the generation of T cell tolerance to Ags present in the intestinal lumen, including those derived from food and the commensal flora (1, 2). The follicle-associated epithelium (FAE)[3] that covers the lymphoid follicles of both Peyer's patches (PP) and isolated lymphoid follicles (ILF) contains specialized epithelial cells known as microfold cells (M cells) that provide a portal for efficient sampling of particulate Ags from the lumen (3, 4). Ags acquired through this major pathway for Ag sampling in the intestine are delivered into intraepithelial pockets within the M cells that lymphocytes and APC access from the subepithelial dome region. The M cell-mediated Ag-sampling pathway has a central role in the development of immune responses to both pathogenic bacteria and commensal bacteria. Production of protective fecal IgA in mice after oral infection with invasive *Salmonella* species requires the presence of PP with M cells (5, 6). In addition, some commensal bacteria internalized through M cells are passed into dendritic cells (DC) that travel with their cargo to the draining mesenteric lymph node, leading to both IgA Ab production and establishment of T cell tolerance (7). M cells also promote the development of T cell tolerance to Ags acquired through the gastrointestinal tract. Targeting OVA to mouse M cells via the reovirus sigma 1 protein resulted in enhanced development of oral tolerance in $CD4^+$ T cells (8). Although most M cells in the small intestine of wild-type mice are localized to the FAE of PP and ILF, occasional villi contain clusters of cells known as villous M cells that exhibit all the major defining characteristics of PP M cells including reactivity with the *Ulex europaeus* agglutinin-I (UEA-I) lectin recognizing α(1,2)-fucose, stubby microvilli, and the capacity to ingest and transcytose particles the size of bacteria (9).

Although the basic functional and ultrastructural features of M cells were initially described over 30 years ago (10), many basic questions about M cell differentiation and function remain unsolved. It has been proposed that specific factors released from the lymphoid microenvironment immediately beneath the FAE have the potential to elicit M cell differentiation in the FAE and promote the function of M cells, but specific signaling mediators with such activity have not been identified to date (11, 12). Debate continues on whether M cells are a distinct lineage arising from crypt stem cells like other differentiated intestinal epithelial cells or whether M cells can instead arise from normal FAE enterocytes with the plasticity to transition into M cells upon encountering the right set of stimuli (13–15).

RANKL (receptor activator of NF-κB ligand) is a member of the TNF superfamily (16) that is also referred to as TNF-related activation-induced cytokine and TNFSF11. Like TNF-α, RANKL is initially synthesized as a transmembrane protein that can be released from the cell surface following cleavage by one of several metalloproteases (17, 18). RANKL signals through its receptor RANK (receptor activator of NF-κB) and a downstream pathway that involves TRAF6 and the activation of NF-κB (19, 20). Osteoprotegerin is a soluble decoy receptor for RANKL that allows for tight regulation of the circulating levels of RANKL (21). A

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[3] Abbreviations used in this paper: FAE, follicle-associated epithelium; PP, Peyer's patch; ILF, isolated lymphoid follicle; M cell, microfold cell; DC, dendritic cell; RANK, receptor activator of NF-κB; RANKL, RANK ligand; mTEC, medullary thymic epithelial cell; GST, glutathione S-transferase; UEA-I, *Ulex europaeus* agglutinin-I; DAPI, 4′,6-diamidino-2-phenylindole.

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