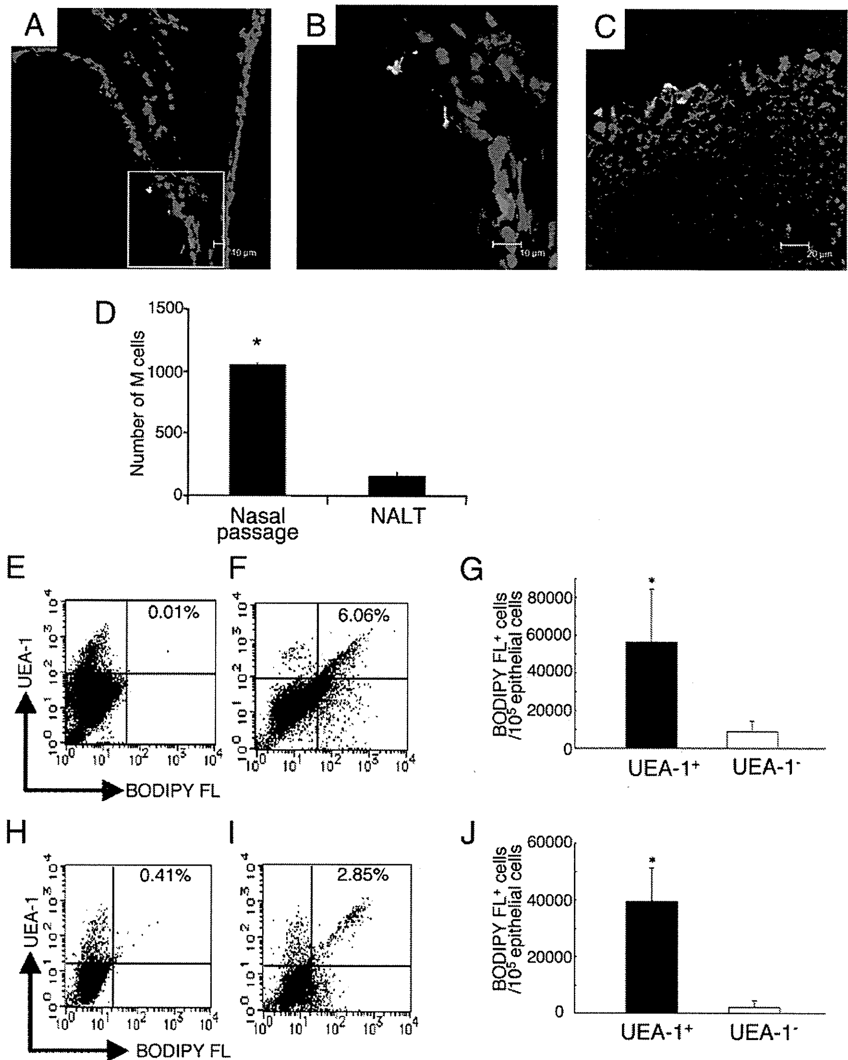

FIGURE 3. Respiratory M cells can take up DQ OVA. *A* and *B*, Immunofluorescence staining of nasal passages in BALB/c mice 30 min after DQ OVA (0.5 mg, green) instillation. Frozen sections of nasal passage were stained with rhodamine–UEA-1 (red) and DAPI (blue). Scale bars, 10 μm. The merged image is shown in *A*. An enlargement of the area in the rectangle in *A* is shown in *B*. These pictures demonstrate DQ OVA uptake by UEA-1^+ respiratory M cells. *C*, UEA-1^+ (red) NALT M cells in BALB/c mice also show an ability to take up DQ OVA (green). Scale bar, 20 μm. The results are representative of seven independent experiments. *D*, The numbers of UEA-1^+WGA$^-$ cells in nasal passages and NALT were quantified. The results are representative of four independent experiments. Flow cytometric analysis of DQ OVA uptake by UEA-1^+ respiratory (*E–G*) and NALT (*H–J*) M cells 30 min after intranasal instillation of PBS (*E*, *H*; control) or DQ OVA (*F*, *I*). *G* and *J*, UEA-1^+ cells showed significantly higher uptake of DQ OVA than did UEA-1^- cells in the nasal passages and NALT. The results are representative of four independent experiments. $^*p < 0.05$.

spiratory epithelium of the nasal passage (Figs. 3*E–G*, 4*D–F*) and NALT (Figs. 3*H–J*, 4*G–I*).

Three-dimensional confocal microscopic analysis demonstrated that UEA-1^+ GFP$^+$ cells, which were sorted from the nasal passages of the mice intranasally infected with GFP-*Salmonella*, had captured and taken up the bacteria (Fig. 4*J*, Supplemental Video 1).

Cluster formation by respiratory M cells and DCs in response to inhaled respiratory pathogens

Because respiratory M cells are capable of capturing bacterial Ag, we considered it important to assess these cells as potential new entry sites for respiratory pathogens such as GAS. Confocal microscopic analysis demonstrated that, after its intranasal instillation, GAS stained with FITC–anti-*Streptococcus* A Ab was taken up by UEA-1^+ respiratory M cells (Fig. 5*B–E*). SEM analysis also revealed the presence of GAS-like microorganisms on the membranes of respiratory M cells after nasal challenge with GAS (Supplemental Fig. 2*A*). As one might expect, GAS were found in UEA-1^+ NALT M cells (Supplemental Fig. 2*B*) as well, confirming a previously reported result (20). Our findings suggest that respiratory M cells act as alternative entry sites for respiratory pathogens.

When we examined the site of invasion by GAS, we noted the presence of CD11c$^+$ DCs underneath the respiratory M cells (Fig. 5). Confocal microscopic analysis of the nasal passage epithelium after intranasal instillation of GAS revealed evidence of the recruitment of DCs, some having contact with the GAS, to the area underneath the respiratory M cells (Fig. 5*B–E*). A few DCs were also observed in the nasal passages of naive mice (Fig. 5*A*); these nasal DCs might preferentially migrate to the area underneath the respiratory M cells to receive Ags from these cells for the initiation of Ag-specific immune responses.

Presence of respiratory M cells in NALT-deficient mice

When we examined the numbers of respiratory M cells in the lymphoid structure-deficient Id$2^{-/-}$ mice (including NALT, NALT-null), the frequency of occurrence of respiratory M cells was comparable to that found in their littermate Id$2^{+/-}$ mice (Fig. 6*A*). This finding suggested that development of respiratory M cells occurred normally under NALT-null or Id2-deficient conditions. Frozen tissue samples were next prepared from NALT-null mice that had received fluorescence-labeled bacteria by intranasal instillation. Immunohistological analysis of these samples revealed the presence of recombinant *Salmonella*-GFP in UEA-1^+ cells from the nasal epithelium of Id$2^{-/-}$ mice. GFP-positive bacteria were also located in the subepithelial region of the nasal passages, suggesting that, in the NALT-null mice, some of the nasally deposited bacteria were taken up by respiratory M cells (Fig. 6*B*, 6*C*). Flow cytometric analysis confirmed the uptake of recombinant *Salmonella*-GFP by UEA-1^+ M cells, with UEA-1^+ cells in the nasal passages of Id$2^{-/-}$ mice showing a significantly higher uptake than UEA-1^- cells (Fig. 6*D–F*).

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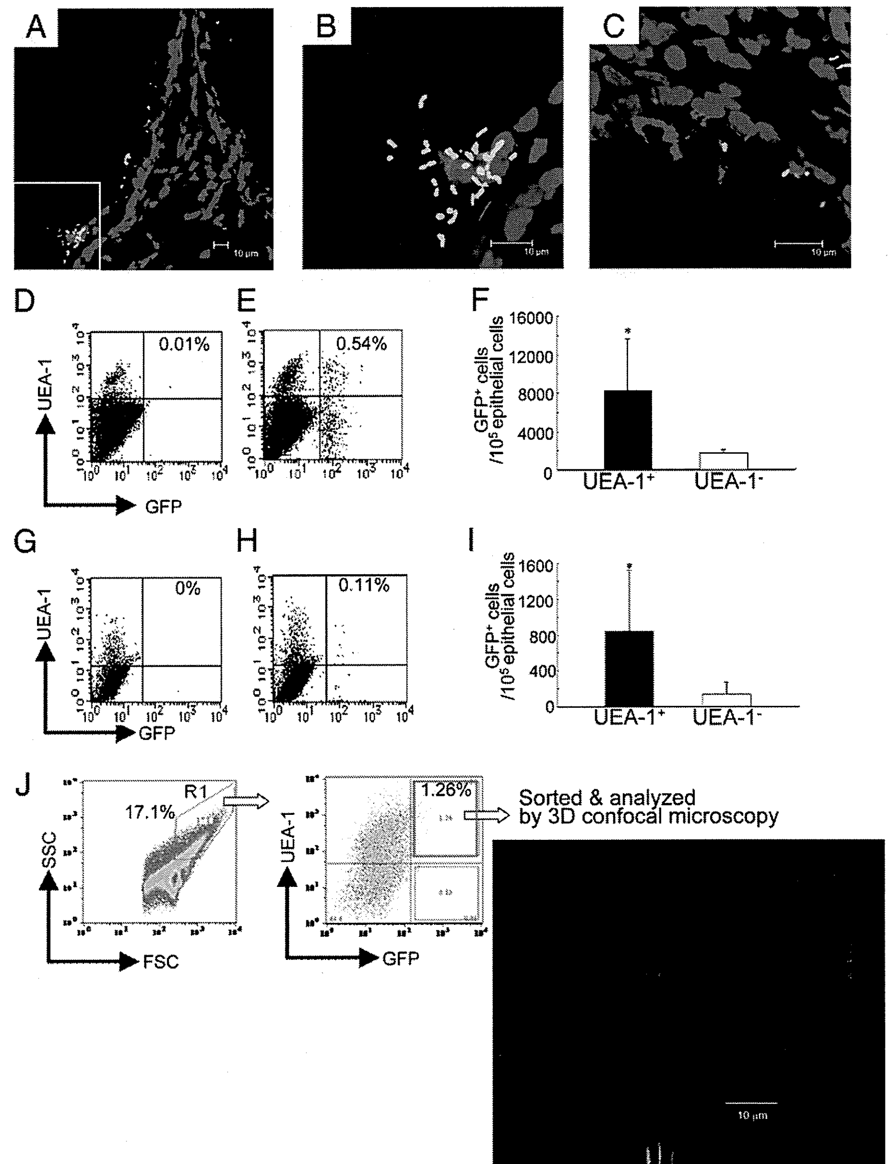

FIGURE 4. Respiratory M cells show an ability to take up recombinant *Salmonella*-GFP. *A* and *B*, Immunofluorescence staining of the nasal passages of BALB/c mice 30 min after GFP-*Salmonella* (5×10^8 CFU, green) instillation. Frozen sections of nasal passage were stained with rhodamine–UEA-1 (red) and DAPI (blue). The merged image is shown in *A*. An enlargement of the area in the rectangle in *A* is shown in *B*. These pictures demonstrate the ability of UEA-1$^+$ respiratory M cells, like UEA-1$^+$ NALT M cells (*C*), to take up GFP-*Salmonella*. The results are representative of six separate experiments. *A–C*, Scale bars, 10 μm. Flow cytometric analysis of GFP-*Salmonella* uptake by UEA-1$^+$ respiratory (*D–F*) and NALT (*G–I*) M cells 30 min after intranasal instillation of PBS (*D*, *G*; control) or GFP-*Salmonella* (*E*, *H*). *F* and *I*, Efficiency of uptake of GFP-*Salmonella* by UEA-1$^+$ cells in both nasal passages and NALT. The data showed UEA-1$^+$ M cells to be significantly more efficient than UEA-1$^-$ epithelial cells at taking up GFP-*Salmonella*. The results are representative of five independent experiments. *J*, Three-dimensional confocal microscopic analysis demonstrated that UEA-1$^+$ GFP$^+$ cells, which were sorted from the nasal passages of mice intranasally infected with GFP-*Salmonella* (green), took up bacteria. Scale bar, 10 μm. The results are representative of three separate experiments. $^*p < 0.05$.

Induction of Ag-specific immune responses in NALT-deficient mice

NALT-null (Id2$^{-/-}$) mice and their littermate Id2$^{+/-}$ mice were intranasally immunized with recombinant *S. typhimurium* BRD 847 expressing a 50-kDa ToxC fragment of tetanus toxin (recombinant *Salmonella*-ToxC) to examine whether Ag sampling via respiratory M cells could induce Ag-specific immune responses in NALT-deficient mice. To eliminate any possible GALT-associated induction of Ag-specific immune responses from the swallowing of bacterial solutions after intranasal immunization, mice were given drinking water containing gentamicin from 1 wk before the immunization to the end of the experiment and were also subjected to intragastric lavage with 500 μl gentamicin solution before and after intranasal immunization. This protocol successfully eliminated the possibility of the intranasally delivered bacteria becoming deposition in the intestine (Supplemental Fig. 1). The titer of TT-specific serum IgG Ab was as high in Id2$^{-/-}$ mice as in Id2$^{+/-}$ mice (Fig. 6*G*). TT-specific IgA Abs were also detected in the nasal secretions and vaginal washes of intranasally immunized NALT-deficient mice (Fig. 6*H*, 6*I*). As expected, TT-specific Abs were not detected in either Id2$^{-/-}$ or Id2$^{+/-}$ mice intranasally immunized with a control recombinant *Salmonella* that did not express the ToxC gene (Fig. 6*G–I*). In addition to the responses to *Salmonella*, GAS-specific immune responses were induced in the absence of NALT in the experiment with Id2$^{-/-}$ mice (Fig. 6*J–L*). These data indicate that the respiratory M cell is an important Ag-sampling site for the induction of Ag-specific local IgA and serum IgG immune responses.

Discussion

In this study, we show the existence of a novel Ag sampling site for inhaled Ags in the upper respiratory epithelium. The murine nasal membrane has been reported to contain four types of epithelium: respiratory, olfactory, transitional, and squamous (21). Most of the respiratory epithelium is located in the lateral and ventral regions of the nasal cavity and is covered with pseudostratified ciliated columnar cells (21). In this study, we were also able to observe a single-layer epithelium on the lateral surfaces of the turbinates, which was comprised exclusively of UEA-1$^+$WGA$^-$ M cells (Fig. 1). These respiratory M cells showed specific reactivity to our previously developed M cell-specific mAb NKM 16-2-4 (12). Because NALT is characterized by follicle-associated epithelium, we first thought that this single-layer epithelium could represent the follicle-associated epithelium of the nasal passage. However,

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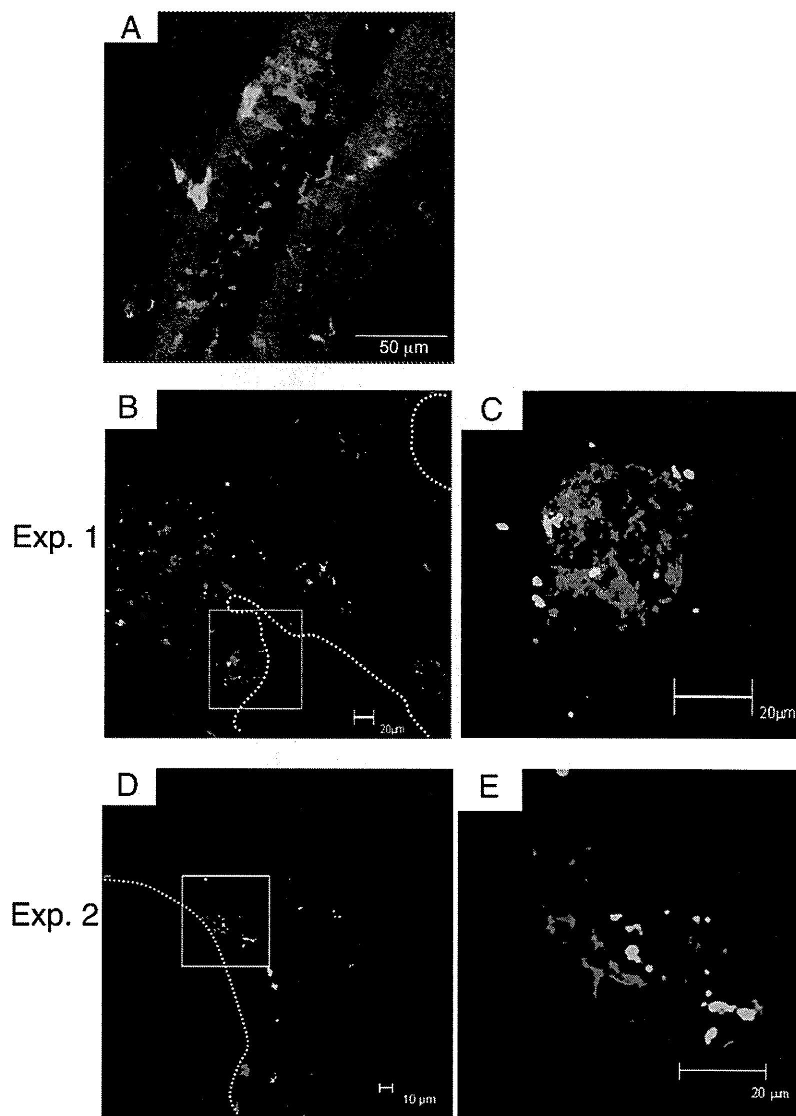

FIGURE 5. Respiratory M cells form clusters with DCs after GAS infection. *A*, Before nasal challenge with GAS, only a few DCs (FITC-CD11c$^+$, green) were associated with UEA-1$^+$ M cells (red) in the nasal passage. Scale bar, 50 μm. *B–E*, Two sets of confocal views of the nasal passage 5 d after intranasal instillation of GAS (Exp. 1 and Exp. 2, respectively). Frozen sections of the nasal passage were stained with FITC–anti-*Streptococcus* A Ab (green), rhodamine–UEA-1 (red), and allophycocyanin-CD11c (blue). These images reveal large numbers of DCs congregated underneath the UEA-1$^+$ respiratory M cells; some of the DCs were closely associated with GAS infiltrated through the UEA-1$^+$ respiratory M cells. *C* and *E* are enlargements of the areas in the squares shown in *B* and *D*, respectively. The results are representative of five independent experiments. *B*, *C*, and *E*, Scale bars, 20 μm; *D*, scale bar, 10 μm.

we ruled out this possibility when we could not find any organized lymphoid structures beneath the single-layer epithelium. The respiratory M cells had most of the classical features of M cells, including a depressed surface covered with short and irregular microvilli. However, TEM analysis revealed that, unlike NALT M cells, they lacked an intraepithelial pocket (Fig. 2). Examination of the numbers of respiratory and NALT M cells per nasal cavity revealed that there were more respiratory M cells than NALT M cells (in general six or seven times more; Fig. 3*D*), suggesting that the respiratory M cell plays a critical role as a gateway for the upper airway.

The anatomical and histological characteristics of the nasal cavity differ markedly between humans and mice. Reflecting this fact, the occurrence of single-layer epithelium also differs between the two species. Murine respiratory epithelium consists of a typical single-layer epithelium with traditional columnar epithelial cells in the turbinate portion of the nasal cavity, whereas pseudostratified columnar epithelium covers the olfactory epithelium (21, 22). In contrast, the traditional single-layer epithelium is not observed in the human nasal cavity, and both the upper respiratory surfaces and the olfactory surfaces are covered by pseudostratified columnar epithelium (23, 24). These differences suggest that the presence of respiratory M cells in the nasal cavity might be a feature unique to the mouse. The presence or absence of respiratory M cells in the human nasal cavity still needs to be carefully examined, and, if these cells are present, their contribution to the uptake of inhaled Ags needs to be investigated in future studies.

Previously, M cells in the lower respiratory tract were found to provide a portal of entry for bacterial pathogens into the lung (25). Our study suggests that the newly identified NALT-independent M cells in the upper respiratory tract provide an alternative portal of entry for nasally inhaled pathogens. The respiratory epithelium comprises three distinct Ag-sampling and/or pathogen-invasion sites: respiratory M cells and NALT M cells in the upper respiratory tract and M cells in the lower respiratory tract. It is interesting to speculate that the nature of the respiratory pathogen may dictate its preferred entry site, with GAS preferentially invading the host via the upper respiratory tract M cells and *Mycobacterium tuberculosis* preferentially invading via the lower respiratory tract M cells. This attractive possibility requires careful examination, and such a line of investigation has been initiated in our laboratory.

Salmonella, a known gastrointestinal pathogen, may have no relevance to the immunological and physiological aspects of Ag uptake by respiratory M cells. However, when used as a live vector for the intranasal delivery of vaccine Ags, attenuated *Salmonella* effectively elicits Ag-specific immune responses (26–29). Pasetti et al. (28) compared intranasal and orogastric immunizations in

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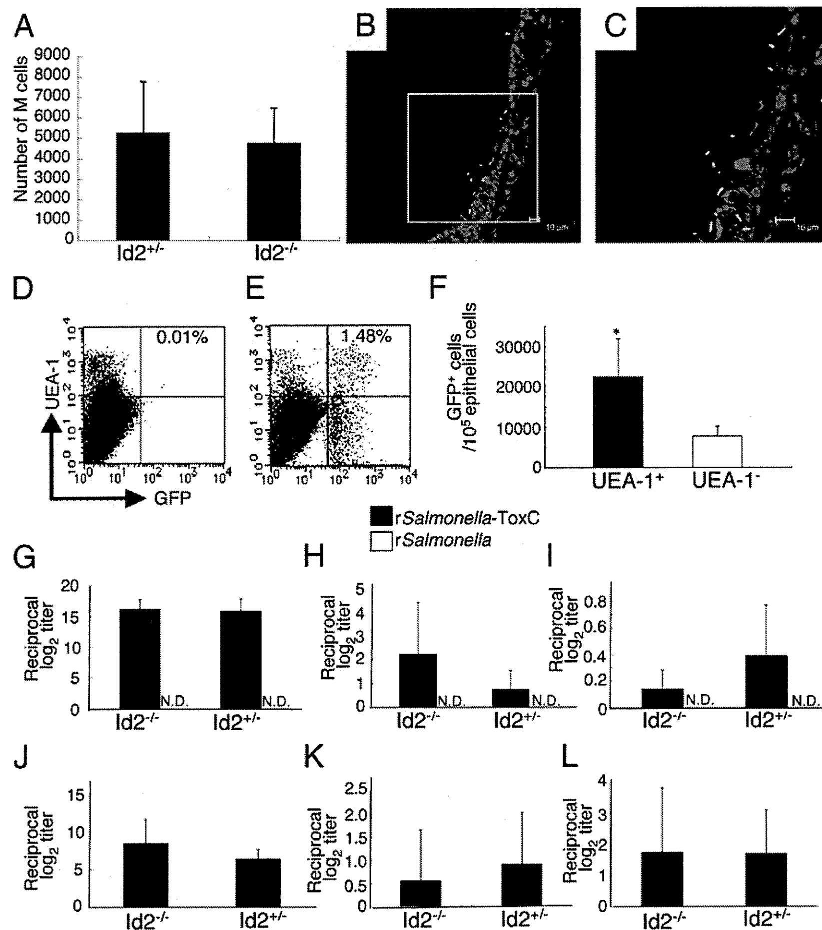

FIGURE 6. Id2$^{-/-}$ mice, which lack NALT, can take up GFP-*Salmonella*, which induce Ag-specific immune responses in UEA-1^{+} respiratory M cells. *A*, The numbers of UEA-1^{+}WGA^{-} cells in nasal passages of Id2$^{-/-}$ and Id2$^{+/-}$ mice were measured. The results are representative of four independent experiments. *B* and *C*, Immunofluorescence staining of nasal passages of Id2$^{-/-}$ mice in which GFP-expressing *Salmonella* (green) had been instilled. Frozen sections of nasal passages were stained with rhodamine–UEA-1 (red) and DAPI (blue). Scale bars, 10 μm. *C* is an enlargement of the area in the square shown in *B*. The results are representative of three independent experiments. *D–F*, Flow cytometric analysis of GFP-*Salmonella* uptake by UEA-1^{+} M cells 30 min after intranasal instillation of PBS (*D*; control) or GFP-*Salmonella* (*E*) in the nasal passages of Id2$^{-/-}$ mice. *F*, Efficiency of uptake by UEA-1^{+} cells in the nasal passages of Id2$^{-/-}$ mice was significantly greater than that by UEA-1^{-} cells. The results are representative of three independent experiments. *G–I*, NALT-deficient (Id2$^{-/-}$) mice and Id2$^{+/-}$ mice were intranasally immunized with recombinant *Salmonella*-ToxC (2.5×10^8 CFU) or recombinant *Salmonella* (2.5×10^8) alone three times at weekly intervals. They were given gentamicin-containing drinking water and also subjected to intragastric lavage with gentamicin solution to eliminate GALT-mediated Ag-specific immune responses. Samples were obtained 7 d after the last intranasal immunization to measure TT-specific Igs by ELISA. Serum IgG (*G*), nasal wash IgA (*H*), vaginal wash IgA (*I*). The results are representative of three independent experiments. *J–L*, As was the case with *Salmonella*, GAS-specific immune responses were induced in the absence of NALT (i.e., in Id2$^{-/-}$ mice), this time by a single intranasal injection of GAS (2×10^8 CFU). Serum IgG (*J*), nasal wash IgA (*K*), vaginal wash IgA (*L*). There were no statistical differences between Id2$^{-/-}$ and Id2$^{+/-}$ mice, as analyzed by the unpaired Mann–Whitney *U* test. The results are representative of five independent experiments. *$p < 0.05$. N.D., not detected.

terms of both Ag-specific immune responses and in vivo distribution of vaccine organisms; they demonstrated that intranasal immunization resulted in greater humoral and cell-mediated immune responses and in the delivery of larger numbers of vaccine organisms to the nasal tissues, lungs, and Peyer's patches. Furthermore, intranasal immunization effectively induces Ag-specific IgA Abs in the reproductive secretions of mice and primates (30, 31). Notably, the levels of Ag-specific IgA Abs in the nasal secretions of NALT-deficient Id2$^{-/-}$ mice were not significantly higher than, or comparable to, those of control tissue-intact mice following intranasal immunization with recombinant *Salmonella* expressing ToxC (Fig. 6*H*) or GAS (Fig. 6*K*), respectively. In contrast, in intranasally immunized NALT-deficient mice, the levels of Ag-specific IgA Abs in remote secretions such as the vaginal wash were not significantly lower than, or comparable to, those in similarly treated tissue-intact mice (Fig. 6*I*, 6*L*). Inasmuch as these results revealed no significant differences between the two groups of intranasally immunized mice, our results at least suggest that respiratory M cells contribute to the induction of Ag-specific immune responses at both local and distant effector sites. However, we still need to carefully examine and compare the contributions of respiratory M cells and NALT M cells in the initiation of Ag-specific IgA Ab responses at local (e.g., airway) and distant (e.g., reproductive tract) effector sites.

In regard to the functional aspects of respiratory M cells, our data demonstrated that the numbers of respiratory M cells that took up OVA were comparable to those of NALT M cells (Fig. 3*G*, 3*J*). In contrast, 10 times more respiratory M cells than NALT M cells took up *Salmonella*; this result suggested that respiratory M cells are more efficient at taking up bacterial (or particulate) Ags than are NALT M cells (Fig. 4*F*, 4*I*). Although we do not have any data regarding the mechanism(s) behind these findings, these results

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suggest that there may be functional differences in, for example, Ag uptake capability, between respiratory M cells and NALT M cells due to possible differences in the expression of bacterial Ag receptors, even though the morphologies and phenotypes of these two subsets of M cells are similar. In support of this possibility, it has been shown that the expression of a GP-2–specific receptor for FimH bacteria is restricted to Peyer's patches and not villous M cells; this situation may be analogous to that of NALT and respiratory M cells (32). Although the molecular mechanisms for the induction of Ag-specific immune responses by intranasal immunization and the efficacy of intranasal inoculation await elucidation, we demonstrated in this paper that respiratory M cells, like NALT M cells, are capable of sampling *Salmonella*, thereby opening a new avenue for the uptake of *Salmonella*-delivered vaccine.

CD18-expressing phagocytes (33) and mucosal DCs (34) are involved in the uptake of pathogens from the lumen of the intestine, but their role in the upper respiratory tract has never been clarified. Moreover, we found no evidence that mucosal DCs take up pathogens from the lumen of the nasal passage by expanding their dendrites into the lumen after nasal challenge with GAS. It was recently shown that intranasal immunization of mice with OVA plus adenovirus vector expressing Flt3 ligand as a mucosal adjuvant selectively increases $CD11b^+$ DC numbers in the nasal passages more effectively than those in NALT and subsequently induces Ag-specific Ab and CTL responses (35). Therefore, we speculated that the induction of immune responses in the murine model of intranasal administration of bacteria (e.g., *Salmonella* and GAS) might depend on the presence of appropriate initial Ag sampling sites associated with M cells, which can internalize the vaccine organisms. In this study, DCs were rarely detected in the subepithelial layer or the epithelial layer of the nasal passage in naive mice (Fig. 5*A*). It is important to note that DCs migrated to the area underneath the respiratory M cells and accumulated there to form cell clusters after exposure to respiratory pathogens (Fig. 5*B–D*). Following mucosal exposure to pathogens, submucosal DCs accumulate underneath infected mucosal epithelium that is not associated with organized lymphoid follicles (36, 37). Furthermore, these Ag-capturing DCs are capable of migrating into the draining lymph nodes (dLNs), where they encounter naive T cells for initial Ag-priming (36, 37). The question of whether DCs resident in the nasal passages migrate to the submucosal area to receive inhaled pathogens taken up via respiratory M cells and then travel to the dLNs (e.g., the cervical lymph nodes) to initiate an Ag-specific immune response remains to be addressed. It is interesting to postulate that respiratory M cells could be alternative airway Ag sampling sites for subsequent processing or presentation by nasal passage DCs, thereby initiating Ag-specific immune responses in the dLNs. In support of this hypothesis, it has been shown that Ag-specific Th cells are generated and found in the NALT and dLNs of mice given GAS intranasally (38). Our current study offers proof in support of this hypothesis by showing that *Salmonella* were effectively taken up by upper respiratory tract M cells in NALT and respiratory M cells and that a live vector-containing vaccine Ag induced Ag-specific immune responses via the nasal route.

We showed that TT-specific serum IgG and nasal wash IgA immune responses after intranasal immunization with recombinant *Salmonella*-ToxC were as high in $Id2^{-/-}$ mice as in $Id2^{+/-}$ mice (Fig. 6*G*, 6*H*) and that the frequency of occurrence of respiratory M cells in $Id2^{-/-}$ mice was comparable to that in their littermate $Id2^{+/-}$ mice (Fig. 6*A*). Generally, as discussed above, submucosal and dermal DCs have been shown to migrate to (or to be located in) the area just beneath infected epithelium and to then migrate into the dLNs after they have captured Ags. The DCs then present the peptides derived from these Ags to naive T cells, which subsequently undergo differentiation to Ag-specific effector T cells (36, 37). It has further been suggested that, rather than the DCs harboring Ag-derived peptides migrating to the systemic compartments, such as spleen and other secondary lymphoid tissues, the effector T cells generated in the dLNs after mucosal or vaginal Ag application migrate to these compartments and initiate Ag-specific immune responses (36).

If the cross-talk system between the airway mucosal and systemic immune compartments is similar to that between the reproductive mucosal and systemic immune compartments, it is unlikely that, in $Id2^{-/-}$ mice, the initiation of Ag-specific immune responses, including the presentation of Ags to naive T cells, occurs through migration of nasal DCs into the spleen after the capture of GAS-Ags by respiratory M cells and DCs. However, we cannot rule out this possibility, because it is possible that the nasal immune system, including the system by which Ags are taken up by respiratory M cells, offers distinct Ag-capture, -processing, and -presentation mechanisms via nasal DCs for the generation and migration of Ag-specific effector T cell and B cells. We have also found B-1 cell populations in the nasal passages (N. Tanaka, S. Fukuyama, T. Nagatake, K. Okada, M. Murata, K. Goda, D-Y. Kim, T. Nochi, S. Sato, J. Kunisawa, T. Kaisho, Y. Kurono, and H. Kiyono, manuscript in preparation), and it is possible that these cells may contribute to the induction of Ag-specific Ig responses without any help from $CD4^+$ T cells. At this stage, this is mere speculation, and the precise mechanism needs to be addressed in the future.

Taken together, these findings led us to conclude that respiratory M cells are effective alternative sampling sites for nasally inhaled bacterial Ags and thus play a key role in the induction of systemic and local mucosal immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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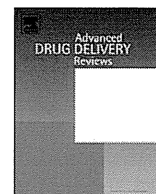

Gut-associated lymphoid tissues for the development of oral vaccines ☆

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ABSTRACT

Oral vaccine has been considered to be a prospective vaccine against many pathogens especially invading across gastrointestinal tracts. One key element of oral vaccine is targeting efficient delivery of antigen to gut-associated lymphoid tissue (GALT), the inductive site in the intestine where antigen-specific immune responses are initiated. Various chemical and biological antigen delivery systems have been developed and some are in clinical trials. In this review, we describe the immunological features of GALT and the current status of antigen delivery system candidates for successful oral vaccine.

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1. Introduction

Despite physical and biological barriers, the gastrointestinal tract is a major route of entry for numerous pathogens. Barriers include epithelial cells (EC) joined firmly by tight junction proteins, brush-border microvilli, and a dense layer of mucin [1]. Antimicrobial peptides, such as defensins produced by ECs and Paneth cells, are additional barrier to provide further protection [2].

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In addition to these barriers, the gastrointestinal tract includes immunological defense system, in particular secretory-immunoglobulin A (IgA) [3], which is predominantly produced at intestinal mucosa by the harmonious interaction between ECs and mucosal lymphocytes and blocks microbial infections by inhibiting adherence of mucosal pathogens at the intestinal lumen to host ECs. Secretory IgA (SIgA) can also neutralize toxins produced by gut pathogens by binding to biologically active sites of toxins.

The immunological characteristics of the gastrointestinal tract have focused attention on the development of effective oral vaccines. Oral vaccination offers several advantages over parenteral vaccination, including needle-free delivery, easy and comfortable administration, and the possibility of self-delivery. Most importantly, oral vaccination can induce both mucosal and systemic immunity, leading to the double layers of protective immune responses [4]. In contrast, parenteral immunization primarily yields a systemic immune response. Therefore, effective oral vaccination could establish a first line of immunological defense in the intestinal tract, a major site of pathogen entry, as well as promote immune surveillance perhaps at other mucosal and systemic sites. One of the major strategies of oral vaccine has been induction of pathogen- or toxin-specific SIgA.

The hostile environment of the gastrointestinal tract (low pH, presence of digestive enzymes, and the detergent activity of bile salts) often makes it difficult to induce protective immune responses by oral vaccination with antigen alone. Additionally, effective oral delivery of antigen to the induction site of the mucosal immune system (e.g., gut-associated lymphoid tissues :GALT) is made difficult by the significant dilution and dispersion of antigen that occurs in the lumen since a total interior area of the intestinal wall is thought to be equivalent to over one tennis coat surface. Further, physical barriers, such as mucus and the tight junctions between the ECs prevent the effective delivery of vaccine antigen. To overcome these obstacles, effort has focused on development of effective antigen delivery systems. In this review, we describe the immunological features of gut-associated lymphoid tissue as the most obvious target site of antigen delivery in the development of oral vaccines. We also describe the current strategies being used to develop versatile antigen delivery systems for efficient oral vaccination.

2. Immunological features of GALT

GALTs comprise several different organized lymphoid structures [5]. Among them, Peyer's patches (PPs) are well characterized as sites for the initiation of intestinal IgA responses. Isolated lymphoid tissue (ILF) is another GALT structure, which is also important in the induction of intestinal IgA responses.

2.1. Peyer's patches (PPs)

PPs are considered to be one of the largest organized lymphoid tissues in the gastrointestinal immune system. There are generally 8 to 10 PPs in the small intestine of mice and hundreds in humans [6]. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as interfollicular region (IFR) (Fig. 1).

Although PPs share some common immunological and micro-architectural features with peripheral secondary lymphoid organs, they are harboring unique features as the mucosa-associated lymphoid tissue [6]. For example, PPs contain efferent but not afferent lymphatics. To compensate, PPs are covered with a specialized epithelial region, termed follicle-associated epithelium (FAE), containing specialized antigen-sampling microfold or membranous cells (M cells). The M cells are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles, and efficient transcytosis activity, allowing the selective and efficient transfer of antigens from the intestinal lumen into PPs (Fig. 2) [7]. Thus, M cells are considered

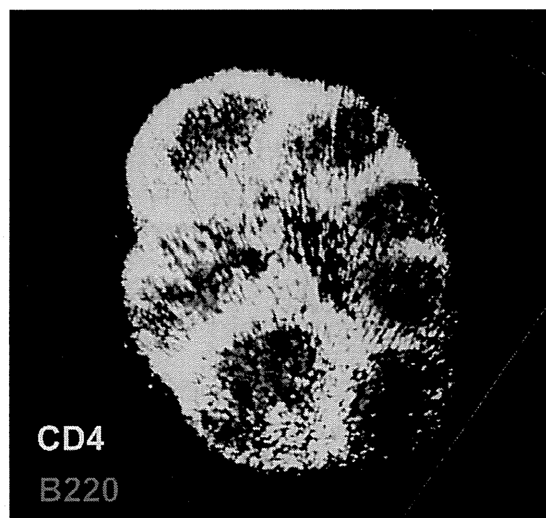

Fig. 1. Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and arboxy SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's patches was observed at the whole tissue level by using macro-confocal microscopy.

to be a professional antigen sampling and gateway cells for the mucosal immune system.

Dendritic cells (DCs) are abundant in the subepithelial dome region (SED) under the FAE, which thus can immediately take up orally encountered antigens from M cells and process and present antigenic peptides to mucosal T and B cells for the initiation of antigen-specific immune responses (Fig. 3). DCs are also found in the IFR. They are composed of at least three distinct subsets: $CD11c^+$ DCs in the SED, $CD8\alpha^+$ DCs in the T cell-rich IFRs, and double-negative DCs in both the SED and IFRs [8]. In addition to antigen presentation, DCs in the intestinal tissues express retinal dehydrogenase, an enzyme that converts vitamin A into retinoic acid. Retinoic acid promotes the preferential homing of activated antigen-specific T and B cells into the intestinal lamina propria by inducing the expression of gut imprinting molecules, such as $\alpha 4\beta 7$ integrin and CCR9 [9,10].

B cells, a major component of PP cells (~75%), are preferentially located in the follicle region (Figs. 1 and 3). Unlike other lymphoid organs, formation of germinal centers (GC) occurs in the PPs even under homeostatic conditions by the continuous stimulation from commensal bacteria, in which leads to the creation of molecular and

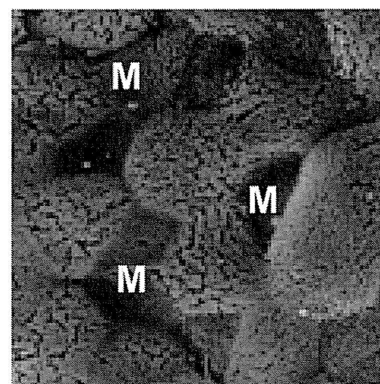

Fig. 2. Scanning electron micrograph of M cells in the Peyer's patches. Scanning electronic microscopy demonstrates that the M cells (indicated as "M") in the Peyer's patches are distinguished from surrounding ECs by their depressed position relative to the ECs, dark brush border, and short microvilli.

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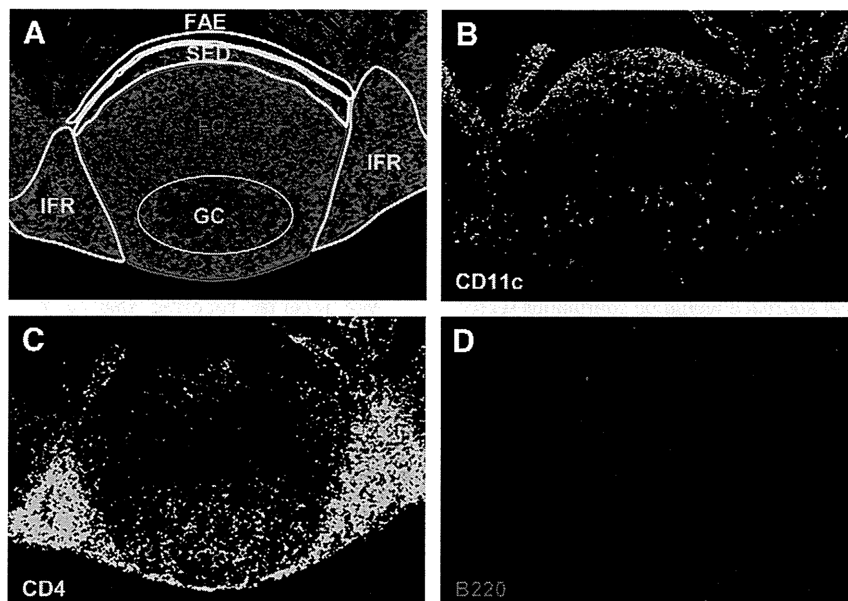

Fig. 3. Distinct cell distribution in the Peyer's patches. Immunohistochemical data on Peyer's patches is shown. (A) Each cell was identified with 4′,6-diamidino-2-phenylindole staining. PP compartments are outlined and labeled as follows: FO, follicle; FAE, follicle-associated epithelium; GC, germinal center; IFR, intrafollicular region; SED subepithelial dome. (B–D) Immunohistochemical staining of PPs for: dendritic cells (anti-CD11c; B), T cells (anti-CD4; C), B cells (anti-B220; D).

cellular environment for class switching of B cells from IgM to IgA (Fig. 3). Thus, PPs contain B cells at several differentiation and maturation stages: IgM$^+$B220$^+$ (~70%), IgM$^+$IgA$^+$B220$^+$ (~1%), IgA$^+$B220$^+$ (~3%), and IgA$^+$B220$^-$ (~0.5%).

Approximately 20% of PP cells are T cells. Some portions of T cells are found in the IFRs of the PPs, which contain mainly naive T cells (Figs. 1 and 3) [11]. In addition to naïve T cells, other T cells exhibit active phenotype, including IFN-γ-producing Th1, IL-4-producing Th2, and IL-10-producing Foxp3$^+$ regulatory T cells [12]. A recent study demonstrated that at least some portions of Foxp3$^+$ regulatory T cells differentiated into follicular helper T cells which facilitate the B cell class switching to IgA$^+$ B cells in the GC [13].

Organogenesis of PPs is initiated in the embryonic stage. In mice, clustering of mesenchymal-lineage VCAM-1$^+$ICAM-1$^+$ PP organizer (PPo) cells starts at the site of tissue anlagen at embryonic days 14–16 [14]. PP inducer (PPi) cell are also key cells that initiate PP organogenesis. PPi cells are a component of lymphoid tissue inducer (LTi) cells that express key transcription factors, Id2 and RORγt, as well as a unique pattern of cell surface markers (IL-7 receptor [IL-7R]$^+$, CD3$^-$, CD4$^+$, CD45$^+$, lymphotoxin [LT] α1β2). The interaction between PPi and PPo cells through the IL-7R and LTβ receptors (LTβR) with corresponding cytokines results in production of lymphoid chemokines such as CXCL13 and CCL19/CCL21 from PPo cells. These chemokines recruit lymphocytes and DCs to form the PP micro-lymphoid structure. Several lines of evidence have demonstrated that the loss of any part of the organogenesis pathways results in the disruption or impairment of PP development [14]. Of note, disruption of the PP organogenesis pathway by blockade of IL-7R and/or LTβR signaling during a limited time period leads to the selective loss of PPs without affecting other lymphoid tissue organogenesis [14]. Experiments with PP-deficient mice showed that they failed to develop antigen-specific immune responses against orally administered particle-form antigens but retained their ability to respond to soluble forms of antigens [15,16], suggesting that PPs play an important role in the induction of antigen-specific immune responses against particulate antigen. The finding may provide a clue for the creation of mucosal antigen delivery vehicle which effectively distributes vaccine to appropriate intestinal inductive lymphoid tissues (e.g., GALT or PPs) covered by FAE containing M cells.

2.2. Isolated lymphoid follicles

Mice selectively deficient in PPs retain certain levels of intestinal IgA responses [15,16]; this finding demonstrates the presence of alternative induction pathways for intestinal IgA production that are independent of PPs. In fact, ILFs were identified as an additional inductive tissue for IgA production. ILFs are located throughout the small intestine as clusters of 100–200 lymphocytes [17]. As for PPs, the formation of ILFs is mediated by the crosstalk between LTi cells and organizer cells. Thus, ILF formation was impaired in RORγt-deficient mice, which lack both PPs and ILFs. When RORγt-deficient mice were reconstituted with RORγt$^+$ LTi, naturally produced intestinal IgA responses were recovered with the newly formed ILFs [18].

ILFs are composed of a single follicle that contains predominantly B cells and some DCs and are covered with a FAE, which contains M cells [17]. In contrast to PPs, ILFs lack T cell-rich IFRs. In agreement with this finding, a recent report indicated that ILFs are a site for T cell-independent IgA production. Indeed, in contrast to PPs, which lack the IgA$^+$ cells in T cell-deficient mice, many IgA$^+$ B cells were still noted in the ILFs of TCR-deficient mice [18]. For the delivery of vaccine antigen to the gut mucosal immune system, an interesting strategy might be the selective delivery of T cell-dependent and -independent antigens to PPs and ILFs, respectively.

3. Antigen-sampling system in the gut

3.1. M cells in the GALT are specialized for antigen sampling

As mentioned above, FAE in the PPs contains M cells that act as a portal for uptake of antigen from the intestinal lumen and transfer into the PPs [19]. Approximately 10% (mouse) and 5% (human) of cells in FAE are M cells [19]. In both mouse and humans, M cells have been shown to harboring some biological and immunological uniqueness that distinguishes them from surrounding ECs. For example, M cells are characterized by short microvilli, a thin glycocalyx, and reduced activity of intracellular lysosomes [19]. In addition, M cells exhibit an intra-pocket structure at basal sites, where lymphocytes and/or antigen-presenting cells including DCs locate. These features allow the M cells to easily take particle-form antigens including microorganisms from the

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lumen and transport them into the PPs without digestion and processing [19]. M cells also show a unique glycosylation pattern. Thus, ulex europaeus (UEA-1) lectin binds α(1,2) fucose residues that are specifically expressed on mouse M cells and Goblet cells [20]. Similarly, sialyl Lewis A antigen recognized by specific antibody (LM112) is a potential candidate for an M cell marker in humans [21]. We recently developed a murine M cell-specific antibody (NKM 16-2-4) [22]. Intriguingly, the antibody also recognized α(1,2) fucose like UEA-1, but did not bind to Goblet cells that are recognized by UEA-1[20], indicating that additional unique glycosylation pattern exists in M cells. Thus, one interesting and novel approach would be continuous search and characterization of glycoprotein modification patterns of FAE cells for the development of glycosylation targeted vaccine delivery system.

In addition to physiological and morphological features, several receptors important for invasion of pathogens and/or uptake of luminal antigens have been identified on M cells. For example, β1 integrin, identified as a receptor for invasin-mediated infection by *Yersinia*, is expressed on M cells [23]. *Salmonella typhimurium* encodes the specific adhesion molecule, long polar fimbria, which targets M cells [24]. Reovirus derived protein σ1 binds to M cells [25]. Recently, glycoprotein 2 (gp2) was found to be expressed specifically on both human and murine M cells; it recognizes FimH, a component of type I pili on bacterial outer membranes, and thus gp2 acts as a receptor for FimH-expressing bacteria such as *Escherichia coli* and *S. Typhimurium* [26,27].

Several key pathways important in the development of M cells were also recently identified [28]. At the cellular level, studies in B cell-deficient mice suggest that B cells play an important role in the M cell development in PPs. B cell-deficient mice had a decreased number of M cells in PPs and adoptive transfer of B cells reversed this phenotype [29]. At the molecular level, the TNF superfamily plays a critical role in the development of M cells. A recent study demonstrated that CD137 (also known as 4-1BB and induced by lymphocyte activation [ILA]) is required for the functionality of M cells. CD137 deficiency thus resulted in a defect in particle transcytosis by M cells [30]. The fact that the ligand of CD137, 4-1BBL, is expressed on B cells and myeloid lineage cells may explain why M cell development is impaired in B cell-deficient mice. In addition to CD137, another TNF receptor superfamily member, receptor activator of nuclear factor κ-B ligand (RANKL), is reported to be involved in M cell differentiation. The number of M cells in FAE of PPs is reduced in mice lacking RANKL or treated with RANKL-specific neutralizing antibody [31]. These findings will likely yield novel strategies to enhance the M cell development and function, resulting in more efficient antigen delivery in the GALT. Thus, M cell development and function regulating molecules may become new generation of mucosal adjuvants for supporting and enhancing antigen-specific immune responses to orally administered vaccine.

3.2. Epithelial cells and villous M cells

Intestinal ECs not only act as a physiological barrier, but also take part in the immunological function of the intestine by the formation of secretary form of immunoglobulin leading to the secretion of IgA and IgM into the intestinal lumen [1]. Reciprocally, IgG, which is involved in the antigen transport system, is transported from the intestinal lumen via the neonatal Fc receptor (FcRn) expressed on the apical surface of ECs [32]. In addition, ECs release exosomes containing antigen bound to MHC class II. The released MHC-bound antigen is thought to induce tolerance, not activation, of antigen-specific T cell responses [33]. This system might be important aspect of the gut immune system for the creation of immunologically quiescence condition at the harsh environment of intestine.

Among ECs in the villous epithelium, we identified M cells sharing similar characteristic with the M cells originally found in the FAE of PPs (or PP M cells) and termed them villous M cells [34]. Villous M cells are thus morphologically similar to M cells in the PPs and are recognized by UEA-1 lectin and M cell-specific NKM16-2-4 antibody, a marker of murine M cells. The specificity for UAE-1 and NKM 16-2-4 antibody suggests that villous M cells most likely harbor identical α(1,2) fucose based glycosylation molecules. Like M cells, villous M cells were capable of taking up *Salmonella*, *Yersinia*, and *Escherichia coli* expressing invasin. In addition, they are found in villous epithelium in PP-deficient mice, which allow them to still induce antigen-specific IgA responses [15,16]. Thus, villous M cells are an alternative antigen-sampling site and can be consider as the additional targeting site for oral vaccine delivery.

We recently reported that M cell-like α(1,2) fucose based glycosylation can be induced on intestinal ECs by environmental stimuli such as colonization with commensal biota, treatment with cholera toxin, or treatment with dextran sodium sulfate and termed these cells as fucosylated ECs (F-ECs) [35]. Although a functional role of F-ECs in the induction of immune responses against intestinal antigens needs further investigation, these findings suggest additional possible strategies to induce F-ECs for the enrichment of antigen-sampling system at the intestinal epithelium to vaccine administered via oral route.

3.3. Intraepithelial DCs

It is also known that the gut immune system is full of antigen-presenting cells including different subsets of DCs [8]. Some DCs are observed in the epithelium of the terminal ileum, where they extend their dendrites into the lumen and thus capable of taking-up intestinal microorganisms. Among the several subsets of DCs, epithelial DCs uniquely express CX3CR1. They penetrate the epithelial layer without disrupting the epithelial barrier connected with highly sophisticated tight junction molecules such as occludin, claudin 1 and zonula occludens 1, and capture luminal bacteria [36,37]. Because of their unique histological positioning at intestinal epithelium, these DCs can be called as "intraepithelial DCs". Unlike other DCs, CX3CR1$^+$ intraepithelial DCs are a non-migratory and gut-resident population, suggesting that the CX3CR1$^+$ population might play a critical role in the initiation or modulation of local immune responses in the intestinal epithelium or lamina propria regions [38]. Thus, these CX3CR1$^+$ DCs resided in the intestinal epithelium could also be useful targeted cell population for oral vaccine delivery.

4. Induction and regulation of IgA-mediated immune responses in the gut

4.1. GALT-mediated induction of IgA responses

A highly integrated sequence of processes of cellular and molecular interaction occurs in the PPs that lead to the initiation of antigen-specific immune responses (Fig. 4). Antigen transport from intestinal lumen by M cells at the FAE of PPs is an initial step for the induction of antigen-specific immune responses after oral immunization. Antigen is then taken up by DCs that are localized in the pocket of M cells or underneath M cells. Resultant up-regulation of CCR7 chemokine receptor expression on the DCs, allows them to move to the T cell region via locally produced corresponding chemokines (CCL19 and CCL21) in the PP or mesenteric lymph nodes and then present the processed peptide antigen for the generation of antigen-specific T cells [39].

Antigen-primed T cells support IgA class switching and somatic hyper mutation of B cells in the GC through antigen-specific interactions, CD40/CD40 ligand interaction, and cytokine expression (e.g., TGF-β, IL-4, and IL-21) [5]. Simultaneously, retinoic acid derived from PP DCs induces the expression on primed T and B cells of the gut-imprinting molecules α4β7 integrin and CCR9 [9,10]. B cells also alter their expression of receptors for other chemokines (e.g., CXCR5 and CCR10) and sphingosine 1-phosphate, thus determining whether they

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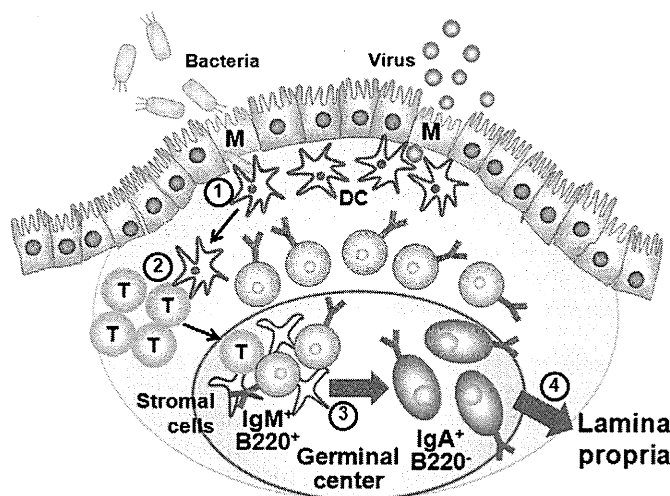

Fig. 4. Sequential processes for initiation of antigen-specific immune responses in Peyer's patches. (1) After transport of antigen by M cells, dendritic cells (DC) take up antigen, and (2) migrate to the T cell region. There, the DCs prime antigen-specific T cells by presenting antigen on MHC molecules and providing co-stimulatory signals. (3) Some of the antigen-primed T cells migrate to the germinal center, where, in coordination with stromal cells and follicular DCs, they induce immunoglobulin class switching and further differentiation of IgM^+ $B220^+$ B cells into IgA^+ $B220^-$ plasmablasts. These germinal center events are dependent on the interaction of CD40 with CD40 ligand, and cytokine activity (in particular TGF-β, IL-4, and IL-21). (4) IgA^+ $B220^-$ plasmablasts modulate their expression of integrins (such as α4β7 integrin) and receptors for chemokines (such as CCR9 and CXCR5) and sphingosine 1-phospahte. These changes promote their emigration from the PPs and trafficking to the intestinal lamina propria where differentiation occurs into plasma cells producing polymeric IgA.

stay in the GC or emigrate from the PPs for the migration to distant effector region (e.g., intestinal lamina propria) [40,41].

After emigration from the PPs, expression of gut-homing molecules (e.g., α4β7 integrin and CCR9/CCR10) on IgA^+ plasmablasts allows them to home to intestinal lamina propria, where IL-5, IL-6, and IL-10 induce terminal differentiation into plasma cells that produce dimeric or polymeric IgA. Polymeric IgA binds polymeric-immunoglobulin receptors expressed on the basal membrane of ECs and is transported to the intestinal lumen as the form of SIgA.

In contrast to events in the PPs, T cell help is not required for the IgA production in the ILFs. As described above, ILFs contain few T cells [17]. A previous study showed that stromal cells could be activated by LTβR-mediated interaction with $ROR\gamma t^+$ LTi and bacterial stimulation through toll-like receptors. This activation resulted in recruitment of DCs and B cells for the subsequent formation of ILFs [18]. Another study demonstrated that simultaneous stimulation of stromal cells with bacteria and retinoic acid induced production of CXCL13, TGF-β, and BAFF and led to preferential generation of IgA^+ B cells [42]. These events occurred in the absence of T cell help [42]. T cell-independent antigens, such as polysaccharides, have been thus considered for use as vaccine antigens [43]. Thus, induction of T cell-independent IgA responses via ILFs could be a novel strategy for the development of oral vaccines.

4.2. GALT-independent IgA production pathway

In addition to conventional B cells (named B-2 cells) which generally located in the organized lymphoid tissues (e.g., PPs), the peritoneal cavity contains large numbers of B-1 cells, another major source of intestinal IgA, especially against T cell-independent antigens [44]. A site for IgA class switching of peritoneal B cells has been elusive, but several lines of evidence indicate the involvement of DCs in the intestinal lamina propria for the creation of class switching molecular and cellular niche. Among the several types of DCs, those that express TNFα and inducible nitric oxide synthase, Tip-DCs, and $TLR5^+$ DCs, induce IgA^+ B cells by producing key molecules, such as APRIL, BAFF, IL-6, and retinoic acid without the involvement of organized lymphoid structure such as PPs [45,46]. However, it was previously reported that lamina propria DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the initiation of intestinal IgA production pathway [47], which was consistent with another finding that DCs in the PPs are responsible for the intestinal IgA synthesis system [48]. Therefore, although it is generally accepted that lamina propria DCs act as antigen-presenting cells for intestinal antigens and are capable of inducing antibody responses, it is still obscure how lamina propria DCs regulate the induction of intestinal IgA and systemic IgG responses.

As ILF-mediate initiated IgA responses, GALT-independent IgA responses are involved in the immune responses against T cell-independent antigens, such as polysaccharides and phosphoryl choline [49]. Since these T cell-independent antigens have been considered as vaccine antigens such as *Streptococcus pneumonia*[43], the use of GALT-independent IgA induction pathway could be an additional strategy for the development of oral vaccines.

5. Application of drug delivery systems to the development of oral vaccines

Antigen delivery is central and key to the development of effective and successful oral vaccines. Particulate antigens appear to be more effective than soluble ones. This phenomenon is at least partially due to protection of the antigen from the harsh conditions of the gastrointestinal environment of digestive tract, such as low pH, detergent effects of bile salts, and extensive proteolytic enzyme activity. In addition, particulate antigens are preferentially taken up in the GALTs, especially by M cells serving as a gateway of the mucosal immune system, thus enhancing their antigenic activity. Several systems have been developed for targeting vaccine antigen selectively to the M cells in the FAE of GALTs.

5.1. Passive transport system

A variety of biodegradable antigen delivery systems have been developed for oral vaccines. These include incorporation of antigens into polymer-based particles (e.g., poly-lactide-co-glycolide-microparticles) [50], liposomes [51], ISCOM [52], and chitosan particles [53]. Their utility as oral delivery vehicles is enhanced by the fact that they are biodegradable and can be formulated for controlled drug release. The effect of particle size on passive targeting to M cells has been evaluated. M cells preferentially take up particles with diameters less than 10 μm whereas a few micrometer- or nanometer-sized particles are taken up by ECs as well as M cells [54]. For example, small poly-lactide microparticles (e.g., 4 μm) in diameter enhanced only plasma IgG responses without IgA responses in the intestine. In contrast, 8–10 μm poly-lactide microparticles enhanced IgA responses in the intestine [55]. These findings suggest that the former size of particles is effectively transported antigen to the systemic immune system (or peripheral lymph nodes) via ECs for the initiation of IgG responses, while the latter sizes are successfully taken up by M cells for the initiation of mucosal IgA antibody responses via PPs. The combination of optimal sizing of capsule is important consideration for the development of oral vaccine which can induce simultaneously both mucosal and systemic protective immunity.

In addition to particle size, modifications to chemical features have been exploited to enhance antigen delivery. For instance, enterocoated-type particles were employed to protect the encapsulated antigen from the acidic environment of the upper part of intestine and to allow rapid release of antigen in the small intestine [56]. An additional example is the use of chemical mucoadhesive molecules (e.g., carboxy vinyl polymer) to elongate particles containing protein antigens, thereby prolonging antigen persistence in the intestine [57]. Liposomes can also

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be made more stable in acid by constructing them with dipalmitoyl phosphatidylserine, dipalmitoyl-phosphatidylcholine, and cholesterol [58,59].

5.2. Use of M cell-specific ligands

In addition to passive one, active delivery of particles to GALT fascinates the induction efficacy of oral vaccines. In this issue, several mucosal antigen delivery systems have been explored that deliver antigen selectively to M cells (Table 1). Lectins have been widely exploited in vaccines to gain or to enhance access of antigen to M cells. The unique reactivity of UEA-1 to M cells allowed the selective and effective delivery of orally administered microparticles or liposomes to murine M cells [60,61]. A similar approach can be taken by using M cell-specific antibodies. NKM16-2-4 recognizing α1,2-fucose-containing carbohydrates. The NKM16-2-4 antibody can be conjugated to vaccine antigen for efficient delivery of antigen to M cells [22]. Thus the targeting to M cells resulted in the induction of antigen-specific IgA antibody responses by the use of low amount of vaccine antigen when compared with the non-targeting form of oral vaccine. Additional studies identified GP2, a receptor for some bacteria expressing Fim(H) [27], as a specific marker of M cells [27] [26]. Because anti-GP2 antibodies have been shown to bind to both murine and human M cells [27], they may be useful for oral antigen delivery in both systems.

The use of organic molecules or peptides that mimic the functional activity of UEA-1 has also been explored to promote efficient delivery of antigen to M cells (Table 1). In these studies, molecules that bound UEA-1 ligands were identified in mixture-based positional scanning synthetic combinatorial libraries or in phage peptide libraries. The former approach revealed that a digalloyl D-Lysine amide construct and a tetragalloyl D-Lysine amide construct bound effectively to M cells; coating of polystyrene particles with these compounds resulted in the selective and efficient delivery of the particles to M cells [62]. The latter approach yielded peptide sequence (YQCSYTMPHPPV) that selectively bound to the M cell-rich SED region of the PP and enhanced the delivery of polystyrene microparticles to M cells [63]. These accumulative evidences suggest that a combination of intestinal friendly characteristics of chemically modified particle and M cell targeting molecule could be a logical strategy for the development of oral vaccine.

5.3. Applying microbial invasion systems to M cell targeting

Another logical approach has been to use components of microbial invasion systems to deliver synthetic particles to M cells (Table 1). Enhanced antigen uptake was achieved by coating polystyrene nanoparticles with *Yersinia*-derived invasin, a ligand for β1 integrins that is expressed on the apical side of M cells [64]. Similarly, mucosal immune responses were significantly increased by mucosal immunization with an antigen coupled to σ1, a protein derived from reoviruses, which are known to be an invading molecule for the virus to enter the M cells [65]. Long polar fimbria (LPF) mediates the binding of *Salmonella* and adherent-invasive *E. coli* to M cells [24,66], but additional pathways appear to exist, as long polar fimbria-deficient *Salmonella* still invade through M cells [67]. In this issue, FimH, the adhesin portion of long polar fimbria, was found to be involved in the binding of FimH(+) *E. coli* and *Salmonella* to M cells [27]. FimH binds to glycoproteins in a mannose-dependent manner and mediates binding to GP2 expressed on M cells [27,68]. Thus, just as for GP2-specific antibodies, FimH is a candidate targeting bacterial molecule for specific delivery of antigen to M cells.

Recently, we employed genetic analyses to identify indigenous commensal bacteria that specifically localized inside of PPs. *Alcaligenes* species, for example, were observed predominantly inside of PPs, in contrast to their absence on the surface as well as other tissues [69]. It has been suggested that at least some component of *Alcaligenes* was taken up by DCs, which induced IL-6 and BAFF expression for the enhancement of IgA production [69]. These findings suggested an interesting possibility that *Alcaligenes* species can be used as a new form of commensal flora based vaccine antigen-delivery micro-vehicle specifically transport vaccine to PPs.

In related to our new observation for the intra-tissue co-habitation of commensal flora, mucosal IgA antibodies have been suggested to play a critical role for guiding and colonizing *Alcaligenes* in PPs since immunoglobulin-deficient mice showed a significant reduction of *Alcaligenes* in the PPs [69]. It is thus possible that antibody-mediated pathway appears to be involved in the uptake of *Alcaligenes* into the PPs [69]. It was previously revealed that immunogloblins preferentially adhere to M cells [70,71], implicating that *Alcaligenes* was taken up by M cells into the PPs via immunoglobulin-mediated pathway. In addition, it was demonstrated that secretory IgA was recognized by DC-SIGN on DCs [72], implicating that M cells and DCs cooperatively use IgA antibody to efficiently enhance the gut immune responses. In line with this, it was previously reported that coating particles with immonoglobulins would target oral vaccines to M cells and consequently enhanced antigen-specific immune responses [73,74].

Table 1
Tools for M cell targeting.

Ligand	Receptor	Reference
UEA-1 lectin	α1,2 fucose	20, 58, 59
Antibody (LM112)	Sialyl Lewis A	21
Antibody (NKM-16-2-4)	α1,2 fucose-containing carbohydrate	20
Antibody (3G7-H9, 2F11-C3)	Glycoprotein 2	26, 27
Digalloyl D-lysine amide	Unknown	60
Tetragalloyl D-lysine amide	Unknown	60
Peptides (YQCSYTMPHPPV)	Unknown	61
σ1 protein (reovirus)	α2,3 sialic acid	25, 63
Invasin (*Yersinia*)	β1 integrin	23, 62
Long Polar fimbriae (*E. coli, Salmonella*)	Unknown	24, 64
FimH (*E. coli, Salmonella*)	Glycoprotein 2	27
IgA	Immunogloblin receptors	71, 72

6. Conclusion

It is generally accepted that mucosal vaccines are an attractive strategy for protecting against many infectious diseases. Recent advances in biomaterial technologies have allowed the development of versatile antigen delivery systems. In addition, significant progress in our understanding of mucosal immunology and M cell biology has enhanced the possibility of targeting mucosal vaccines to the mucosal antigen-sampling and presenting system including M cells, DCs and ECs. Furthermore, because immunological environment in the intestinal tract is dominantly quiescent by several lines of regulatory/suppressor system to maintain the immunological homeostasis in order to deal with the harsh environment of intestine, we also have to consider the development of mucosal adjuvant/modulator to temporary break the immunological suppression for the initiation of antigen-specific positive responses. Thus, integration of the all knowledge gained in the biomaterial, immunological, and cellular biological fields should facilitate the development of a new generation of mucosal vaccines.

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A Pivotal Role of Vitamin B9 in the Maintenance of Regulatory T Cells *In Vitro* and *In Vivo*

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Abstract

Dietary factors regulate immunological function, but the underlying mechanisms remain elusive. Here we show that vitamin B9 is a survival factor for regulatory T (Treg) cells expressing high levels of vitamin B9 receptor (folate receptor 4). In vitamin B9-reduced condition *in vitro*, Treg cells could be differentiated from naïve T cells but failed to survive. The impaired survival of Treg cells was associated with decreased expression of anti-apoptotic Bcl2 and independent of IL-2. *In vivo* depletion of dietary vitamin B9 resulted in the reduction of Treg cells in the small intestine, a site for the absorption of dietary vitamin B9. These findings provide a new link between diet and the immune system, which could maintain the immunological homeostasis in the intestine.

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Introduction

To achieve immunosurveillance and immunological homeostasis at the interface between the interior and exterior of the gastrointestinal tract, the intestinal immune system tightly balances states of immune activation and quiescence [1]. Thus, gastrointestinal tissues contain numerous kinds of T cells, such as Th1, Th2, Th17, forkhead box P3 (Foxp3)$^+$ regulatory T (Treg) cells, IL-10–producing Foxp3$^-$ T regulatory type 1 cells, and T cells expressing $\gamma\delta$ T cell receptor, which together create the appropriate immunological environment.

Th17 and Treg cells are observed most frequently in the intestine, and their preferential differentiation is achieved by a unique cytokine environment created by transforming growth factor β (TGF-β), IL-6, and IL-23 [2]. In addition to these host-derived factors, the development and function of the immune system are influenced by crosstalk with environmental factors [3]. For example, stimulation by segmented filamentous bacteria results in the preferential induction of Th17 cells, whereas colonic Treg cells are induced by crosstalk between epithelial cells and Clostridium clusters IV and XIVa [4,5,6].

Nutritional molecules are also considered to be essential environmental factors for the development, maintenance, and regulation of gut immune responses. Thus, deficient or inappropriate nutritional intake increases the risk of infectious, allergic, and inflammatory diseases [7,8]. Among various dietary factors, vitamins are important participants in the regulation of immune responses. For example, vitamin A is converted into retinoic acid (RA) by gut-associated dendritic cells; RA induces the expression of gut-homing molecules (e.g., α4β7 integrin and CCR9) on activated T and B cells [9,10] and promotes the preferential differentiation of Treg cells and the simultaneous inhibition of Th17 cells [11,12,13,14]. Vitamin B6 is required for the metabolic pathway of sphingosine 1-phosphate, a lipid mediator that regulates cell trafficking [15]; disruption of vitamin B6 function results in aberrant T-cell differentiation and cell trafficking in both systemic and intestinal compartments [16,17,18].

Vitamin B9 (also known as folate and folic acid) is a water-soluble vitamin derived from both diet and commensal bacteria [19]. Vitamin B9 is essential for the synthesis, replication, and repair of nucleotides for DNA and RNA and is thus required for cell proliferation and survival [20]. Methotrexate (MTX) acts as a vitamin B9 antagonist and blocks vitamin B9–mediated nucleotide synthesis, making MTX useful as an anti-tumor [21] and anti-rheumatoid arthritis agent [22]. Vitamin B9 deficiency also reduces the proliferative responses of lymphocytes and natural killer cell activity [23,24]. Additionally, the vitamin B9 receptor folate receptor 4 (FR4) is both a marker of Treg cells and is immunologically functional [25]; however, how it functions in the intestinal immune system is largely unknown. In this study, we examined the role of vitamin B9 in the regulation of Treg cell *in vitro* and *in vivo*.

Materials and Methods

Mice and experimental treatment

Female Balb/c mice (7–9 wk of age) were purchased from Japan Clea (Tokyo, Japan). Vitamin B9–deficient and control

diets composed of chemically defined materials (Oriental Yeast, Tokyo, Japan) were used within 3 months. All animals were maintained in the experimental animal facility at the University of Tokyo, and the experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines (Approval #20–28).

Lymphocyte isolation

Lymphocytes were isolated from the lamina propria (LP), as previously described [18,26]. Briefly, lymphocytes were isolated from dissected PPs by enzymatic dissociation using collagenase (Wako, Osaka, Japan). To isolate lymphocytes from the LP of jejunum/duodenum, PPs were removed and the remaining intestinal tissue was cut into 2-cm pieces and stirred in RPMI 1640 medium containing 1 mM EDTA and 2% fetal calf serum (FCS). The tissue pieces were then stirred in 0.5 (for small intestine) or 1.0 (for large intestine) mg/mL collagenase, and the dissociated cells were subjected to centrifugation through a discontinuous Percoll gradient. Lymphocytes were isolated at the interface between the 40% and 75% Percoll layers.

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as previously described [18,26]. Cells were pre-incubated with anti-CD16/32 antibodies and then stained with fluorescent antibodies specific for CD4, ICOS, and GITR (BD Biosciences, San Jose, CA) and FR4 (Biolegend). A Via-probe solution (BD Biosciences) was used to discriminate between dead and living cells. Intracellular staining of Foxp3 (eBioscience, San Diego, CA), phosphorylated STAT5, Ki67 and Bcl2 (BD Biosciences) was performed in accordance with the manufacturers' instructions. Flow cytometry and cell sorting were carried out using the FACSCantoII and FACSAria systems (BD Biosciences), respectively.

Vitamin B9 measurement

To measure vitamin B9 concentrations, intestinal washes were collected by washing 12 cm of jejunum/duodenum or whole colon with 1 mL of PBS. The vitamin B9 concentration in intestinal washes and serum was measured with a RIDASCREEN enzyme immunoassay kit (R-Biopharm AG, Darmstadt, Germany) in accordance with the manufacturer's instructions. To measure the amounts of intracellular vitamin B9, 5×10^6 purified cells were washed twice with PBS, and a cell lysate was obtained by homogenizing cells in PBS containing 0.01% NP-40. After cell debris was removed by centrifugation, vitamin B9 amounts in the supernatant were measured with a RIDASCREEN enzyme immunoassay kit.

In vitro culture

For the induction of Treg cells from naïve T cells, $CD62L^{hi}CD4^+$ naïve T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody and 1 μg/mL of an anti-CD28 antibody (BD Biosciences) plus 2 ng/mL of human TGF-β (PeproTech, Rocky Hill, NJ) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS. To examine the maintenance of differentiated Treg cells, purified $CD25^+CD4^+$ T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody with or without 1000 units/mL of IL-2 (Peprotech) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS in the presence or absence of 100 nM MTX.

Statistics

Results were compared with the Student's *t*-test by using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was established at $P<0.05$.

Results

Vitamin B9 is required for the survival of $Foxp3^+$ Treg cells

$Foxp3^+$ Treg cells express high levels of FR4, which is essential for their maintenance [25]. We therefore examined whether vitamin B9 is required for the differentiation of Treg cells from naïve T cells, the survival of differentiated Treg cells, or both. To address this, we initially performed an *in vitro* T-cell differentiation assay. Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in complete or vitamin B9–reduced medium. Although a small amount of vitamin B9 is supplied from fetal calf serum (FCS) even in vitamin B9–null medium (0.2 ppb, compared with 25 ppb in normal medium), the total cell number was decreased in the condition with reduced vitamin B9 compared to the control; however, $Foxp3^+$ Treg cells were generated at a normal frequency (Fig. 1A).

To investigate the effects of vitamin B9 on differentiated Treg cells, we cultured $CD25^+$ Treg cells with anti-CD3 antibodies. The total cell number was significantly lower in the vitamin B9–reduced condition than in the control condition (Fig. 1B). The reduction in cell number occurred predominantly among the $Foxp3^+CD4^+$ Treg cells (Fig. 1B). The reduction of $FR4^{hi}Foxp3^+$ T cells was dependent on the dose of vitamin B9 (Fig. 1C).

We then measured the expression of Ki67 and anti-apoptotic Bcl-2 to investigate whether decreased number of $Foxp3^+CD4^+$ Treg cells in vitamin B9–reduced medium was due to the defects of cell proliferation, survival, or both. We found that both Ki67 and Bcl2 were decreased in $Foxp3^+CD4^+$ Treg cells cultured in vitamin B9 vitamin B9–reduced medium, but magnitude of Bcl2 reduction was higher than Ki67 reduction (Fig. 2A and B). These findings suggest that vitamin B9 is preferentially but not exclusively required for the survival of Treg cells *in vitro*.

Vitamin B9 carrier-mediated pathway is not specifically involved in the survival of Treg cells

Because vitamin B9 is highly hydrophilic, mammalian cells must actively mediate the entry of vitamin B9 into cells by carrier- or receptor-mediated pathways [27]. Carriers include the proton-coupled folate transporter and the reduced folate carrier [27]. To examine whether a carrier-mediated pathway is involved in maintaining Treg cells, we employed MTX, an antagonist of vitamin B9 that is transported mainly via the reduced folate carrier and rarely via folate receptors [28,29]. MTX treatment reduced the numbers of both Treg and non-Treg cells (Fig. 3), suggesting that the carrier-mediated pathway does not specifically maintain Treg cells.

Vitamin B9 is an IL-2–independent survival factor for Treg cells

Treg cells could vigorously proliferate in some circumstances (e.g., antigen-specific activation through their highly sensitive TCR signaling [30] and IL-2-mediated activation [31]), which led to a hypothesis that Treg cells simply require large amounts of vitamin B9 as a source of nucleotides, and thus Treg cells might express FR4 as an additional means of acquiring vitamin B9. If so, $FR4^{hi}$ Treg cells should contain a larger amount of vitamin B9 in the intracellular compartments; however, the amount of intracel-

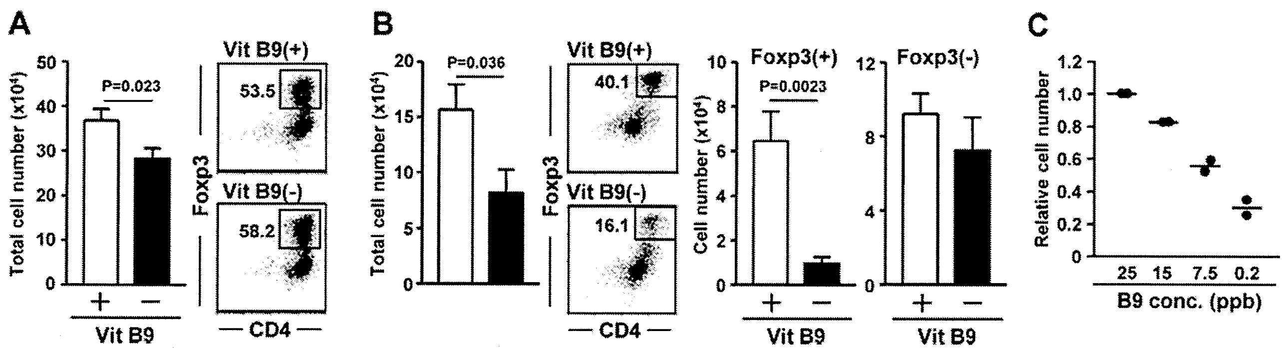

Figure 1. Requirement of vitamin B9 for the maintenance of Treg cells. (A) Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in the presence of normal [Vit B9(+)] or reduced [Vit B9(−)] amounts of vitamin B9. After 4 days, total cell numbers were calculated, and the differentiation into $Foxp3^+$ Treg cells was examined by flow cytometry. Data are means ± SEM (n = 4). (B) $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Cont or B9(−) medium. The frequencies of $Foxp3^+$ and $Foxp3^-CD4^+$ T cells (B) were determined by flow cytometry. Cell numbers were calculated using the total cell number and flow cytometric data. Data are means ± SEM (n = 6). (C) Experiments similar to that shown in (B) were performed with different concentrations of vitamin B9. The relative cell number of $Foxp3^+$ Treg cells is expressed as a ratio to the cell number in control medium. The values and means are indicated with dots and lines, respectively. Similar results were obtained from 2 independent experiments.
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lular vitamin B9 was equivalent between $FR4^{hi}$ Treg and $FR4^{low/-}$ non-Treg cells (Fig. 4A). Thus, FR4 might have an additional specific function for the survival of Treg cells.

IL-2 stimulation enhance the survival of Treg cells [31,32,33]. The FR4-mediated vitamin B9 signal might undergo crosstalk with IL-2-mediated signaling to maintain the survival of $FR4^{hi}Foxp3^+$ Treg cells. To test this, Treg cells were cultured with an anti-CD3 antibody together with IL-2. Although the absolute cell numbers were low in the reduced vitamin B9 condition, the magnitude of the IL-2-mediated enhancement of Treg cell growth was similar in the control and vitamin B9-reduced conditions (Fig. 4B). Consistent with this finding, comparable expression of phosphorylated STAT5 was noted in the control and vitamin B9-reduced conditions (Fig. 4C).

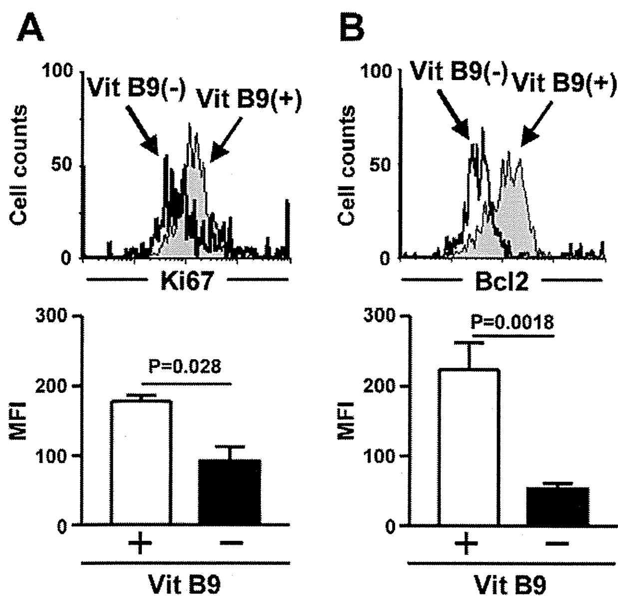

Figure 2. Vitamin B9 is essential for the survival of Treg cells. $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Vit B9(+) or Vit B9(−) medium. The expression of Ki67 (A) and Bcl2 (B) in $Foxp3^+CD4^+$ T cells were determined by flow cytometry (top panels) and graphs show the means fluorescent intensity (MFI; bottom panels). Data are means ± SD (n = 3). Data are representative of 4 independent experiments.
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Dietary vitamin B9 maintains $Foxp3^+$ Treg cells in the small intestine

To examine whether vitamin B9 affects Treg cells *in vivo*, we maintained mice on a vitamin B9–depleted diet for 8 wk. Mice maintained with vitamin B9(−) diet showed less vitamin B9 in the small-intestinal wash than controls (Fig. 5A). In contrast, the amounts of vitamin B9 in the large-intestinal wash and serum were not different in those mice (Fig. 5A), presumably due to vitamin B9 production from commensal bacteria [19].

We then focused on Treg cells in the mice maintained with vitamin B9(−) diet. Consistent with our *in vitro* data, the small intestines of mice maintained with vitamin B9(−) diet had fewer $Foxp3^+$ Treg cells than those of control mice (p = 0.018), and there was no statistical difference (p = 0.3022) in the number of $Foxp3^-CD4^+$ non-Treg cells (Fig. 5B). The number of Treg and

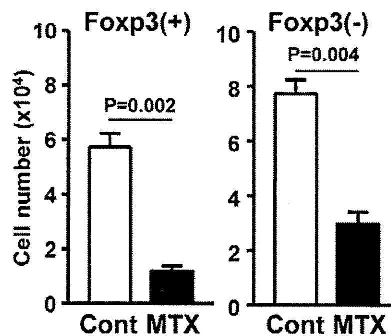

Figure 3. Vitamin B9 carrier-mediated pathway is not specific pathway in the maintenance of T cell survival. $CD25^+$ $CD4^+$ T cells were cultured with an anti-CD3 antibody in complete medium containing 100 nM methotrexate (MTX), and the frequency and absolute cell numbers of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells were determined. Data are means ± SEM (n = 4). Data are representative of two independent experiments.
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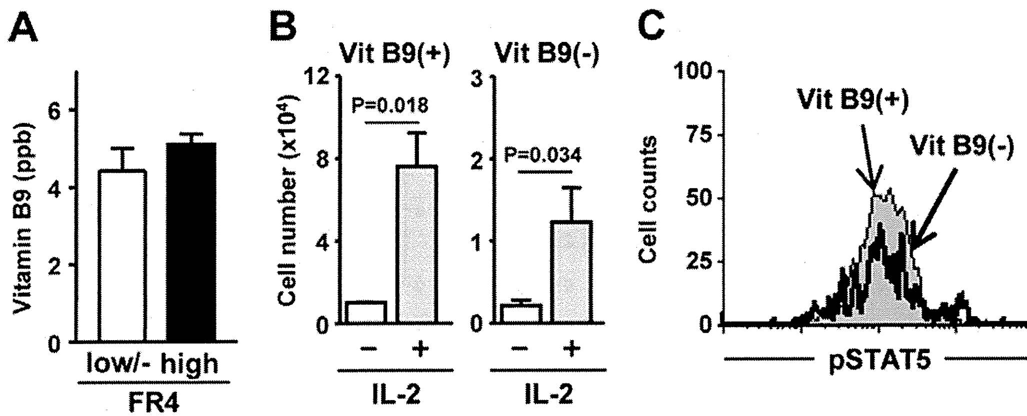

Figure 4. Vitamin B9 is IL-2-independent survival factor for Treg cells. (A) The amounts of intracellular vitamin B9 were measured using purified $CD4^{+}FR4^{hi}$ Treg or $CD4^{+}FR4^{low/-}$ non-Treg cells. Data are means ± SEM (n = 4). (B, C) Experiments similar to those shown in Fig. 1B were performed in the presence of anti-CD3 antibody stimulation with or without IL-2 stimulation. Cell number of $Foxp3^{+}CD4^{+}$ T cells (B) and the expression of phosphorylated STAT5 (pSTAT5) in $Foxp3^{+}CD4^{+}$ T cells (C) were determined. Data in (B) are means ± SEM (n = 6). Similar results were obtained from 3 separate experiments.
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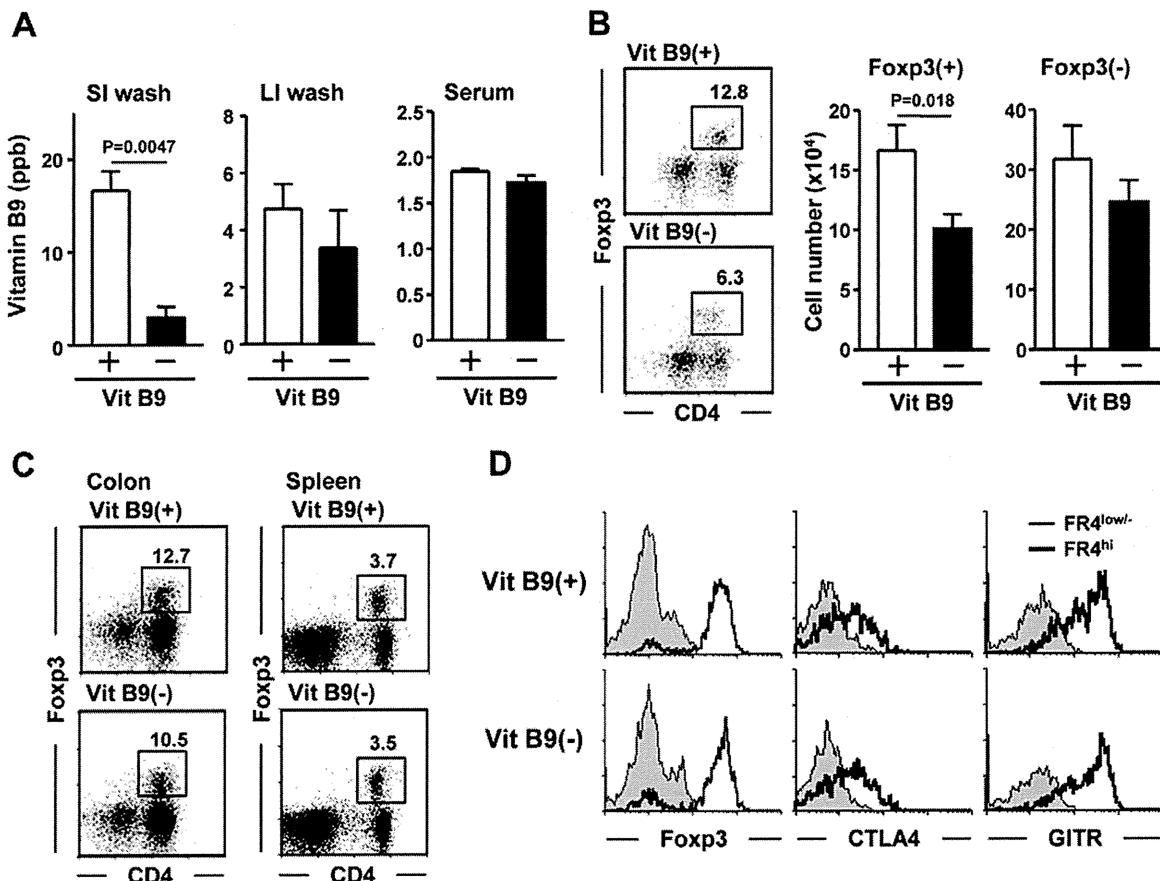

Figure 5. Depletion of dietary vitamin B9 selectively reduces Treg cells in the small intestine. Mice were maintained on a control [Vit B9(+)] or vitamin B9-depleted [Vit B9(−)] diet for 8 wk. (A) Vitamin B9 concentrations were measured in intestinal washes of the small intestine (SI), large intestine (LI), and serum. The data are mean ± SEM (n = 6). (B, C) The frequency and cell numbers of $Foxp3^{+}$ and $Foxp3^{-}$ $CD4^{+}$ T cells in the small intestine (B), colon, and spleen (C) were calculated using the total cell number and flow cytometric data (mean ± SEM, n = 6). (D) Flow cytometric analysis was performed to determine the expression levels of Foxp3, CTLA4, and GITR on the surface of $FR4^{low/-}$ (thin line) and $FR4^{hi}$ (thick line) $CD4^{+}$ T cells in the LP. Similar results were obtained from 3 separate experiments.
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non-Treg cells was not significantly changed in the colon and spleen of mice maintained with vitamin B9(−) diet (Fig. 5C), which could be explained by the similar concentration of vitamin B9 in the large-intestinal washes and sera of both groups of mice. We also found that Foxp3 and the inhibitory molecules CTLA4 and GITR, which are specifically expressed on Treg cells, were comparable between those mice (Fig. 5D).

Discussion

We have shown that vitamin B9 is crucial for the maintenance of Treg cells. Intriguingly, vitamin B9 was required for the survival of differentiated Treg cells, but was not necessary for the differentiation of naïve T cells into Treg cells. This selective effect of vitamin B9 on Treg cells is opposite to the effect of RA, a vitamin A metabolite, which enhances the differentiation of naïve T cells into Treg cells [11,12,13,14]. RA also induces the expression of gut-homing molecules (e.g., $\alpha4\beta7$ integrin and CCR9) on B and T cells activated by gut dendritic cells [9,10]. Because CCR9 was expressed normally on Treg cells in the LP of mice maintained with vitamin B9(−) diet (data not shown), the deficiency of dietary vitamin B9 did not affect the RA-mediated expression of gut-homing molecules and, predictably, the induction of Treg cells in the small intestine.

Treatment with the vitamin B9 antagonist MTX affected survival of both Treg cells and non-Treg cells, suggesting that the carrier-mediated pathway maintains sufficient amounts of intracellular vitamin B9 for cell survival regardless of the T-cell subset. The indiscriminate effects of MTX could be explained by the ubiquitous expression of the folate carrier [29,34]. As the mechanism of FR4-mediated Treg-cell maintenance, we considered initially that the proliferative activity of Treg cells could require large amounts of vitamin B9 as a source of nucleotides for DNA and RNA. However, the amounts of intracellular vitamin B9 were identical between Treg and non-Treg cells, implying that FR4 specifically recognizes extracellular vitamin B9 for the maintenance of Treg cell survival, consistent with a report that FR4 expressed on Treg cells contributes to their immune function and survival [25]. Additionally, the specific biological functions of vitamin B9 receptors (FR1, FR2, and FR4) have been predicted on the basis of their ~70% amino acid sequence identity, but the expression of each receptor is rigidly restricted, with narrow tissue and cell specificity [35,36]. Because FR1, FR2, and FR4 are glycosyl phosphatidylinositol–anchored proteins [37], adapter molecules may assist FR4 in the maintenance of Treg cell survival. We found that vitamin B9/FR4 was not associated with IL-2–mediated signaling in Treg cells. We will continue to study how FR4-mediated vitamin B9 regulates the survival of Treg cells.

Mammals must obtain vitamin B9 from the diet or from commensal bacteria. The absorption of vitamin B9 from the diet occurs mainly in the small intestine, whereas the uptake of microbial vitamin B9 predominantly occurs in the colon [38]. This explains why depletion of dietary vitamin B9 specifically decreased Treg cells in the small intestine, but not in the colon. It has been proposed that bacterial vitamin B9 absorbed in the colon affects the vitamin B9 status of the host [39,40], which may explain the lack of changes in vitamin B9 in the serum and splenic Treg cells in mice maintained with vitamin B9(−) diet. *Bifidobacterium*, one of the most important genera of commensal bacteria to be used as a probiotic, is well-studied as a vitamin B9 producer [41], and colonic Treg cells are specifically induced by immunological crosstalk with commensal bacteria, especially *Clostridium* clusters IV and XIVa [5]. Although whether *Clostridium* clusters IV and XIVa produce vitamin B9 remains unclear, our current findings suggest that vitamin B9 is an essential survival factor for Treg cells and, in vivo situation, diet vitamin B9 establishes an immunological network in the maintenance of Treg cells specifically in the small intestine.

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Author Contributions

Conceived and designed the experiments: JK. Performed the experiments: JK EH II. Analyzed the data: JK EH II. Wrote the paper: JK HK.

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