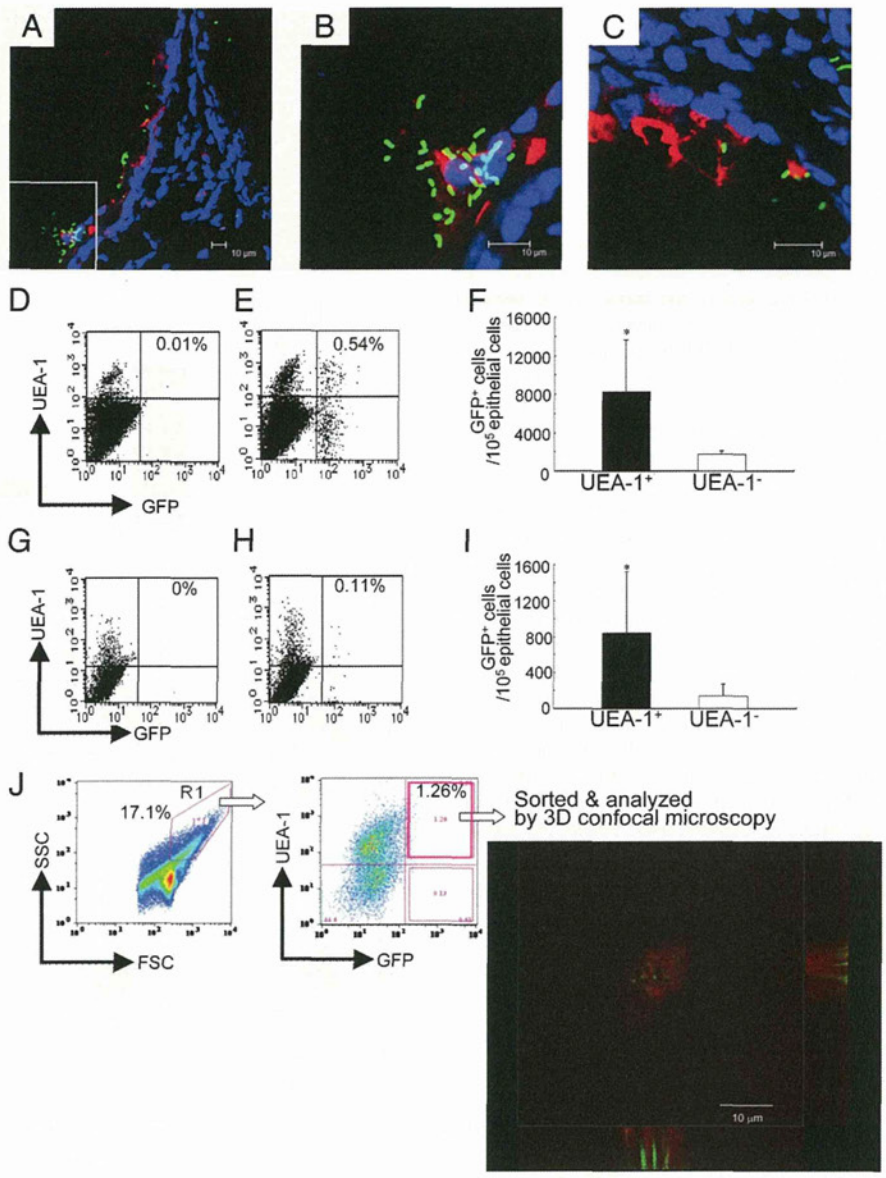

FIGURE 4. Respiratory M cells show an ability to take up recombinant *Salmonella*-GFP. *A* and *B*, Immunofluorescence staining of the nasal passages of BALB/c mice 30 min after GFP-*Salmonella* (5×10^8 CFU, green) instillation. Frozen sections of nasal passage were stained with rhodamine–UEA-1 (red) 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,

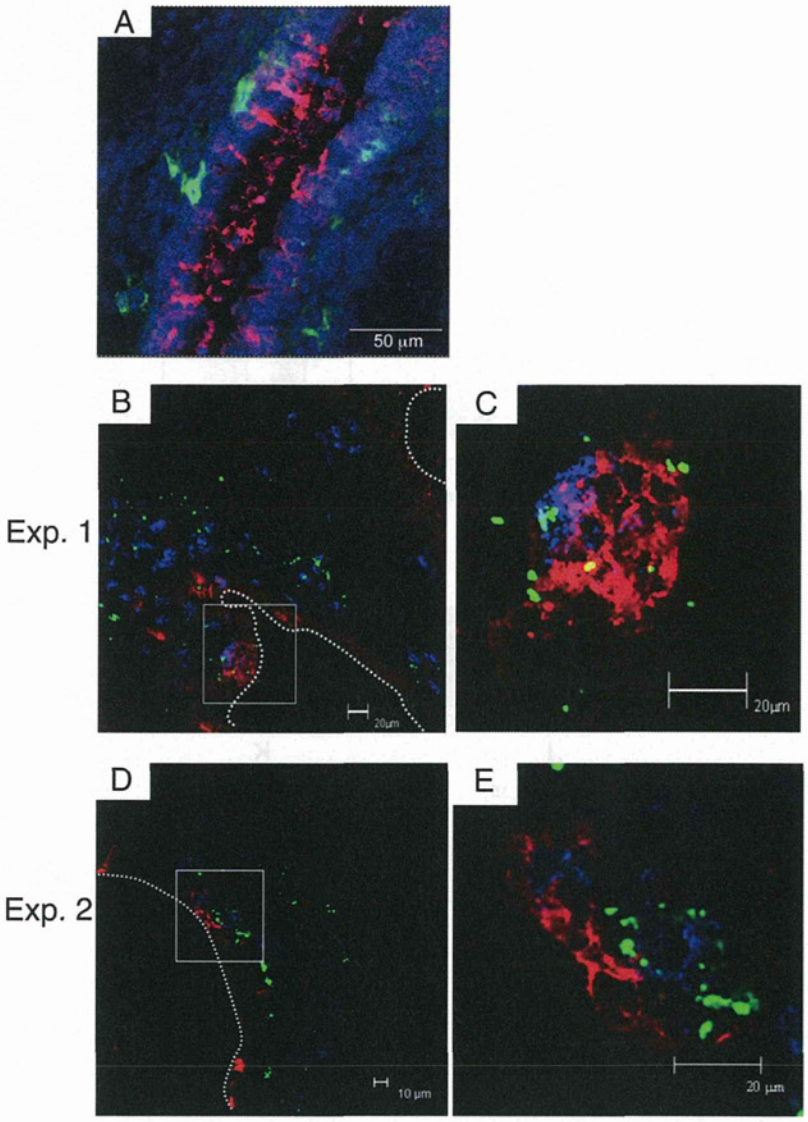

FIGURE 5. Respiratory M cells form clusters with DCs after GAS infection. *A*, Before nasal challenge with GAS, only a few DCs (FITC-CD11c$^+$, green) were associated with UEA-1$^+$ M cells (red) in the nasal passage. Scale bar, 50 μm. *B–E*, Two sets of confocal views of the nasal passage 5 d after 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

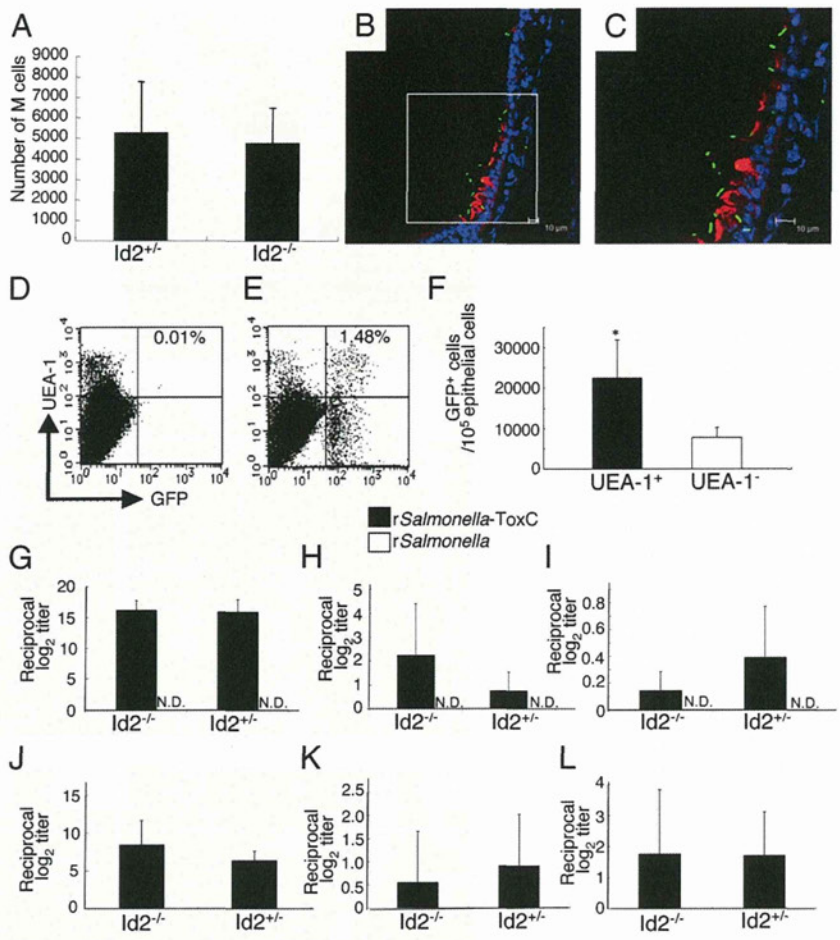

FIGURE 6. Id2$^{-/-}$ mice, which lack NALT, can take up GFP-*Salmonella*, which induce Ag-specific immune responses in UEA-1^{+} respiratory M cells. *A*, The numbers of UEA-1^{+}WGA^{-} cells in nasal passages of Id2$^{-/-}$ and Id2$^{+/-}$ mice were measured. The results are representative of four 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

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.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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Epithelial cell microRNAs in gut immunity

Yoshiyuki Goto & Hiroshi Kiyono

MicroRNAs regulate many biological functions. Research now indicates that intestinal epithelial microRNAs might also regulate the differentiation of goblet cells and promote T helper type 2 immune responses to parasite infection.

The intestine is a unique organ that is constantly exposed to an almost limitless array of foreign antigens, including not only innocuous food-derived materials and harmless commensal microbes but also pathogenic bacteria, viruses and parasites. The establishment and maintenance of appropriate immune quiescence or activity in the gut mucosa requires highly sophisticated immunological regulatory systems generated by the cooperative interaction between intestinal epithelial cells and mucosal cells of the immune response. Among numerous regulatory molecules, microRNAs (miRNAs) have received much attention as a newly identified family of regulators in animal and plant cells. Although the critical function of these small RNAs is suggested to contribute to the establishment of immunological homeostasis at mucosal sites, only limited information on this is available at present. Now a study by Biton *et al.* in this issue of *Nature Immunology* reports on how intestinal epithelial miRNAs might regulate the differentiation of goblet cells and the associated development of antiparasitic T helper type 2 (T_H2) immunity[1].

The intestine is covered by an epithelial monolayer comprising four main types of intestinal epithelial cells: columnar epithelial cells, paneth cells, endocrine cells and goblet cells[2]. These epithelial cells form the first physical barrier that separates the host from the external environment. In addition, epithelial cells also detect stimuli from luminal antigens and transmit signals to the various innate and acquired types of mucosal cells of the immune response for the initiation of active or quiescent immune responses. Epithelial cells and mucosal cells of the immune response thus tightly regulate each other, and this crosstalk results in intestinal immunological homeostasis. For example, thymic stromal lymphopoietin (TSLP) produced by epithelial cells limits the production of proinflammatory cytokines by dendritic cells[3], whereas the proliferation and differentiation of epithelial cells is controlled by epithelial cell growth factors such as keratinocyte growth factor generated from intestinal T cells[4]. Further, bilateral regulation of intestinal T_H1 cells versus T_H2 cells and of cells of the T_H17 subset versus regulatory T cells has a critical role in gut immunological homeostasis. Whereas the differentiation of T_H17 cells and regulatory T cells is regulated by commensal bacteria[5–7], the nature of the various pathogens influences the outcome of T_H1- and T_H2-mediated immunity; for example, helminths tend to drive T_H2 development[8]. Helminth-derived antigens are taken up by mucosal dendritic cells and basophils, which leads to the differentiation of T_H2 cells from naive T_H0 cells[8]. T_H2 cytokines such as interleukin 4(IL-4), IL-5 and IL-13 accelerate helminth-specific T_H2-biased immunity[8]. It should be remembered that epithelial cells occupy a physically and perhaps immunologically intermediate position between the parasites and gut mucosal immunity. Epithelial cell–derived TSLP thus promotes T_H2 immune responses by 'educating' intestinal dendritic cells during infection[3]. However, the precise cellular and molecular mechanisms by which epithelial cells establish gut T_H2 immunity are still not fully understood.

The maturation of miRNA occurs via a series of steps. Two main RNase III endonucleases, Drosha and Dicer, are involved in this process. Primary miRNA is processed by Drosha and precursor miRNA is cleaved by Dicer to produce mature and functional miRNA[9]. The inhibitory function of mature miRNA is mediated by two different pathways: the degradation of mRNA by binding of miRNA to the 3′ untranslated region of mRNA, or direct repression of the translational process[9]. Many biological processes are regulated by miRNA, including cell survival, differentiation and homeostasis; furthermore, specific miRNAs regulate the differentiation of intestinal epithelial cells[10].

Goblet cells are of a secretory epithelial lineage that produces carbohydrate-rich mucus to exclude foreign antigens, including pathogens. Although a published report has shown that intestinal epithelial cell–specific deletion of Dicer1 leads to fewer goblet cells, the miRNA targets or the mechanism by which miRNAs affect these changes were not identified[10]. Biton and colleagues have now used mice with inducible intestinal epithelial cell–specific deficiency in *Dicer1* (*Dicer1*$^{\Delta gut}$) to investigate this[1]. Consistent with the results of the previously published study[10], they find fewer colonic goblet cells in *Dicer1*$^{\Delta gut}$ mice. The authors further identify a specific miRNA, miR-375, that probably inhibits translation of the gene encoding KLF5, an antagonist of the goblet cell–differentiation factor KLF4 (**Fig. 1a**); this therefore suggests that miR-375 probably supports the differentiation of goblet cells by targeting an otherwise repressive transcription factor. In addition, they demonstrate that *Dicer1*$^{\Delta gut}$ mice have lower expression of T_H2 cytokines such as IL-4, IL-5 and IL-13, which leads to enhanced susceptibility to infection caused by the mouse helminth parasite *Trichuris muris*. They also identify IL-13 as a possible cytokine that induces miR-375 in intestinal epithelial cells, at least *in vitro*. They provide evidence that IL-13 induces miR-375, KLF4 upregulation and eventual differentiation of goblet cells (**Fig. 1a**). This IL-13

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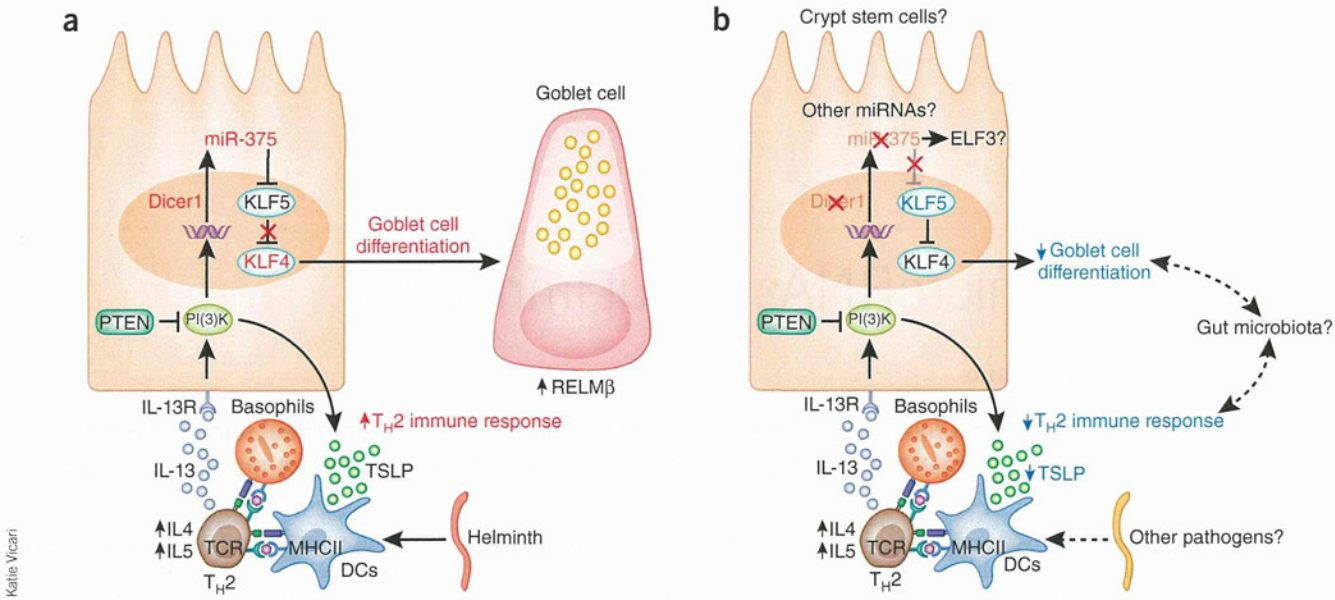

Figure 1 MicroRNAs regulate intestinal epithelial differentiation and T_H2 immune responses. (**a**) Regulation of intestinal innate and acquired immunity by Dicer and miRNA. Dicer1 and miR-375 inhibit KLF5, a known antagonist of KLF4 that promotes the differentiation of goblet cells via KLF4. Helminth infection induces TH2 cytokines, especially IL-13, which leads to epithelial expression of miR-375 and goblet-cell maturation via PI(3)K. Moreover, miR-375 also induces TSLP to accelerate TH2 immune responses to parasite infections. (**b**) Depletion of Dicer1 or miR-375 results in fewer goblet cells and diminished T_H2 responses. On the basis of new findings now reported[1], additional issues have been raised in miRNA-mediated gut immunity. Addressing several unanswered questions will provide fuller understanding of the role of miRNA in establishing optimized mucosal immunity: Does IL-13 directly induce miR-375 in epithelial cells and/or crypt stem cells? Do other miRNAs regulate the differentiation of goblet cells (or other epithelial cells)? Is the gut microbiota involved in the induction and regulation of miRNA expression either for active or quiescent immunity? Are other miRNAs involved in the generation of optimal protective immunity to various pathogens? PTEN, PI(3)K-pathway inhibitory phosphatase; IL-13R, IL-13 receptor; TCR, T cell antigen receptor; MHCII, major histocompatibility complex class II; RELMβ, TH2 antiparasitic cytokine.

is presumably supplied by T_H2 cells because T cell–deficient nude mice have much lower expression of miR-375. Moreover, PI(3)K, a signaling molecule downstream of the IL-13 receptor, mediates miR-375 expression and the differentiation of goblet cells (**Fig. 1a**). IL-13-mediated expression of miR-375, furthermore, leads to TSLP production, which indicates a potential mechanism by which miR-375 could drive an appropriately balanced T_H2 feed-forward loop. On the basis of these results, the authors suggest that the epithelial cell-specific miRNA miR-375 directs the differentiation of goblet cells and the promotion of antiparasitic T_H2 immune responses (**Fig. 1a**). As miR-375 expression is very high in the human intestine[11], mucosal expression of this particular miRNA might also be important in the regulation of intestinal homeostasis and protection against parasite infection in humans.

Several interesting questions are raised by the results reported by Biton *et al.*[1] (**Fig. 1b**). For example, whether the target of miR-375 is solely KLF5 or whether there are other targets relevant to the differentiation of goblet cells and/or gut immunological homeostasis still needs to be clarified. In addition to goblet cell–inducing KLF4, another transcription factor, ELF3, also seems to enhance the differentiation of goblet cells[2]. In support of the contention that other targets may be involved, Biton *et al.* find that deletion of Dicer1 results in more severe impairment of the differentiation of goblet cells than does germline deficiency in miR-375 alone[1]. A more comprehensive analysis of mice with (ideally gut-inducible) knockout of miR-375 or specific antagonism of this miRNA *in vivo* would help delineate its role in gut immunity. It is thus important to elucidate further how miR-375 and probably other miRNAs and their associated targets interact and cooperatively regulate the differentiation of goblet cells.

Goblet cells are constitutively present in normal mice, whereas greater numbers of goblet cells are induced after parasite infection[12]; this suggests that two types of goblet cells develop in the intestinal epithelium: naturally occurring goblet cells and inducible goblet cells. Although ~16% of all epithelial cells in the colon are naturally occurring goblet cells, a limited number of these cells are present in the small intestine, especially the duodenum (~4%)[2]. Although Biton *et al.* find that the differentiation of naturally occurring goblet cells seems to be dependent on miRNA[1], it is possible that IL-13 is a master regulator of this, or that other as-yet-unidentified molecules, including other T_H2-type cytokines, are involved in the differentiation and localization of both types of goblet cell and mucosal expression of miR-375 in the intestine. Moreover, similar to other columnar epithelial cells and endocrine cells, goblet cells are also differentiated from villous crypt stem cells[1]. Does IL-13 produced only by T_H2 cells neighboring crypt stem cells drive miR-375 expression and subsequent differentiation of goblet cells, or do dispersed T_H2 cells and/or other subsets of T cells also have such functions involving epithelial miRNA–mediated cell differentiation and immunity? Answering these questions will help elucidate how miRNAs direct the differentiation of crypt stem cells and the specificity of the miRNAs required for intestinal homeostasis.

During parasite infection, one of the main immunologically relevant functions of epithelial miRNA seems to be TSLP induction and the promotion of T_H2 responses (**Fig. 1a**). In addition to the differentiation of goblet cells, which seems to skew toward T_H2 antiparasitic immune responses, whether other epithelial miRNAs regulate T_H1 and/or T_H2 responses requires further analysis. In addition, defective mucous secretion caused by goblet cell hypoplasia could lead to aberrant

composition and localization of the gut microflora and thereby diminish mucosal T_H2 responses (**Fig. 1b**). It will therefore be important to examine whether the gut microbiota regulates intestinal miRNAs involved in the development of mucosal homeostasis (**Fig. 1b**). The role of mucosa-associated miRNA in the differentiation of epithelial cells and regulation of the mucosal immune system is only just beginning to be explored. Further investigation should open the door to the development of innovative mucosal miRNA–targeted treatments and the diagnosis of pathogenic mucosal conditions such as allergy, inflammatory bowel diseases and colon cancer, as well as infection by bacteria, viruses and parasites.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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AIDing the pursuit of IgA diversity

Kang Chen & Andrea Cerutti

Direct evaluation of the contribution of somatic hypermutation (SHM) to mucosal immunity has been hampered by the lack of models able to dissociate SHM from class-switch recombination, which are both dependent on the cytidine deaminase AID. A new mouse AID model now demonstrates the critical role of SHM in the control of gut bacteria.

The diversification of immunoglobulin genes is critical for the generation of immune protection. This is particularly true at mucosal sites such as the intestine, which is populated by a large community of commensal bacteria. Mature B cells generate immunoglobulin gene diversity by undergoing somatic hypermutation (SHM) and class-switch recombination (CSR) in the germinal center of lymphoid follicles. SHM introduces point mutations into recombined variable-diversity-joining (V(D)J) exons that encode the antigen-binding V region of immunoglobulins, thereby providing the structural correlate for the selection of high-affinity immunoglobulin mutants by antigen[1]. In contrast, CSR replaces the μ-chain constant region (C_μ) exon, which encodes immunoglobulin M (IgM), with C_γ, C_α or C_ε exons, which encode IgG, IgA or IgE, thereby providing immunoglobulins with new effector functions without changing their specificity for antigen[1]. Both SHM and CSR require the DNA-editing enzyme AID (activation-induced cytidine deaminase)[2]. Because of this common reliance on AID and hence the difficulty in dissociating SHM from CSR in mice that lack AID, the specific contribution of SHM to mucosal immunity has remained elusive. In this issue of *Nature Immunology*, Wei *et al.* use a mouse model that expresses an SHM-defective but CSR-competent AID molecule to show that SHM is critical for the generation of homeostasis and immunity in the intestine by B cells[3].

The interaction between the intestinal mucosa and commensals has been in the limelight in the past decade because of its huge relevance to the biology of both health and disease states. It is now well recognized that commensals establish a symbiotic relationship with the host in the intestine, as they process otherwise indigestible food components, synthesize essential vitamins, stimulate the maturation of the immune system, and form an ecological niche that restricts the growth of pathogenic species[4]. Conversely, the host provides commensals with a habitat rich in energy derived from the processing

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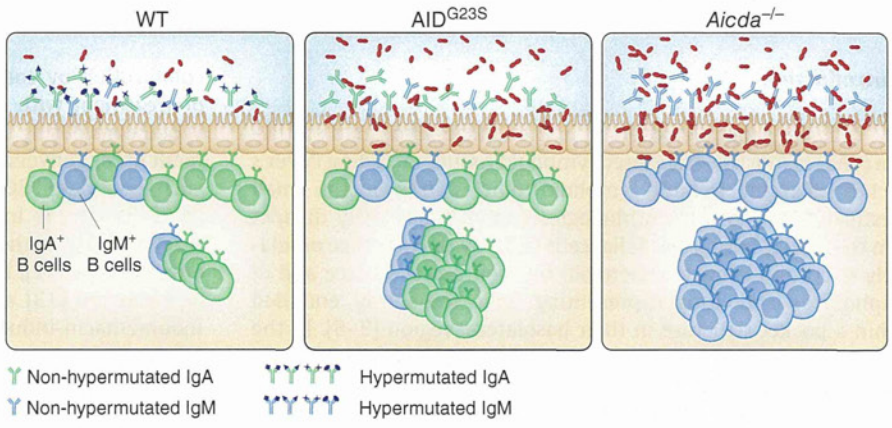

Figure 1 Lack of SHM impairs mucosal homeostasis and immunity. In wild-type (WT) mice (left), AID-mediated antibody diversification via CSR and SHM generates a diversified mucosal repertoire comprising IgA (green) and, to a lesser extent, IgM (blue), which regulates the composition of the local commensal microbiota. Mice expressing the CSR-competent but SHM-defective mutant AIDG^{23S} molecule (middle) produce normal amounts of unmutated IgA and IgM, which cannot efficiently recognize the intestinal microflora, thereby causing aberrant expansion of and more epithelial adhesion of certain bacterial species. More penetration of these bacteria across the epithelial barrier drives the hyperactivation of mucosal lymphoid follicles, including Peyer's patches. Similar but more profound abnormalities are present in AID-deficient (*Aicda*$^{-/-}$) mice (right), which show profound defects in both CSR and SHM and hence express only unmutated IgM.

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Distinct fucosylation of M cells and epithelial cells by Fut1 and Fut2, respectively, in response to intestinal environmental stress

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ABSTRACT

The intestinal epithelium contains columnar epithelial cells (ECs) and M cells, and fucosylation of the apical surface of ECs and M cells is involved in distinguishing the two populations and in their response to commensal flora and environmental stress. Here, we show that fucosylated ECs (F-ECs) were induced in the mouse small intestine by the pro-inflammatory agents dextran sodium sulfate and indomethacin, in addition to an enteropathogen derived cholera toxin. Although F-ECs showed specificity for the M cell-markers, lectin *Ulex europaeus* agglutinin-1 and our monoclonal antibody NKM 16-2-4, these cells also retained EC-phenotypes including an affinity for the EC-marker lectin wheat germ agglutinin. Interestingly, fucosylation of Peyer's patch M cells and F-ECs was distinctly regulated by α(1,2)fucosyltransferase Fut1 and Fut2, respectively. These results indicate that Fut2-mediated F-ECs share M cell-related fucosylated molecules but maintain distinctive EC characteristics, Fut1 is, therefore, a reliable marker for M cells.

1. Introduction

M cells are generally observed in the follicle-associated epithelium (FAE) of mucosa-associated lymphoid tissues including Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) in the small intestine [1,2], and are morphologically and functionally distinct from their neighboring epithelial cells (ECs) by the presence of relatively short and irregular microvilli on their apical surface and of lymphocytes and antigen-presenting cells frequently enfolded within a pocket structure in their basolateral region [3–5]. In the small intestine of mice, the expression of α(1,2)fucose is believed to be a reliable marker of M cells, because lectin *Ulex europaeus* agglutinin-1 (UEA-1), which has an affinity for α(1,2)fucose, was found to bind exclusively to M cells in the PPs [6,7]. Subsequently, we could found M cells located within the non-FAE-associated small intestinal villous epithelium by utilizing an affinity of UEA-1 [8].

Interestingly, α(1,2)fucosylation is also induced in ileal villous ECs by a variety of intestinal environmental stresses (IES) such as colonization by commensal bacteria, weaning, mechanical injury or treatment with chemicals inhibiting protein synthesis [9–11]. When considering the possible involvement of IES in the development of (or conversion to) M cells, it is reasonable to postulate that such fucosylated ECs form a subset of M cells, because the number of PP M cells is increased rapidly and transiently by alteration from specific pathogen-free (SPF) conditions to a conventional environment [12], by interaction with bacteria such as *Salmonella typhimurium* [13] and *Streptococcus pneumoniae* [14] and during indomethacin-induced ileitis [15]. Like PP M cells, villous M cells might also be induced (or converted) by IES, because a higher frequency of villous M cells is observed in the terminal ileum, which is enriched for commensal bacteria [16]. Recently, we found that some ECs underwent α(1,2)fucosylation in the small intestinal villous epithelium when a mucosal adjuvant cholera toxin (CT) derived from a well known enteropathogen *Vibrio cholerae* was orally administered into mice, and that these cells, in part, shared the same gene expression profile as PP M cells; we previously designated them "M-like cells" [17].

In mice, α(1,2)fucosyltransferase Fut1 and Fut2 are the enzymes responsible for catalyzing an α(1,2) linkage of fucose to terminal β-galactoside, and Fut2 is involved in the IES-associated fucosylation

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whereas little is known about Fut1 in the intestine [11,18–20]. In this study, we aimed to elucidate the biological characteristics of ECs that shared the α(1,2)fucose modification with M cells focusing on the fucosylation mechanism, in the hope of better understanding of whether ECs can be reprogrammed into M (or M-like) cells in response to IES.

2. Materials and methods

2.1. Mice

BALB/c and C57BL/6J mice were purchased from SLC (Shizuoka, Japan). Fut1-null and Fut2-null mice (C57BL/6J background) were generated as previously described [21]. These mice were maintained under SPF conditions and used in experiments at 6–9 weeks old. All animal experiments were approved by the Animal Care and Use Committee of The University of Tokyo.

2.2. Lectins and antibodies

The following lectins and antibodies were used for flow cytometry (FCM) and confocal laser scanning microscopy (CLSM): PE-conjugated UEA-1 (UEA-1-PE; Biogenesis, England, UK), tetramethylrhodamine isothiocyanate (TRITC)-conjugated UEA-1 (Vector Laboratories, Burlingame, CA), Alexa Fluor 633-conjugated wheat germ agglutinin (WGA-AF633; Molecular Probes, Eugene, OR), FITC-conjugated NKM 16-2-4 mAb (NKM 16-2-4-FITC) [22], APC-conjugated anti-mouse CD45 mAb (anti-CD45-APC; BD Biosciences, San Jose, CA).

2.3. Alteration of the intestinal environment

A mucosal adjuvant, CT (List Biologic Laboratories, Campbell, CA), and two pro-inflammatory agents, dextran sodium sulfate (DSS, m.w. 36,000–50,000; ICN Biomedicals, Irvine, CA) and indomethacin (Sigma–Aldrich, St. Louis, MO), were used as stress-inducing agents to alter the intestinal environment of mice as described previously [17,23,24] (see Supplementary information).

2.4. Preparation of intestinal epithelial cells for FCM

The small intestinal epithelium was dissociated by a mechanical procedure as described previously [17]. The mononuclear cells were stained with NKM 16-2-4-FITC, UEA-1-PE and anti-CD45-APC and dead cells were stained with 7-aminoactinomycin D (BD Biosciences). Fluorescently labeled cells were analyzed and, if necessary, sort-purified using a FACSAria flow cytometer (BD Biosciences) (see Supplementary information).

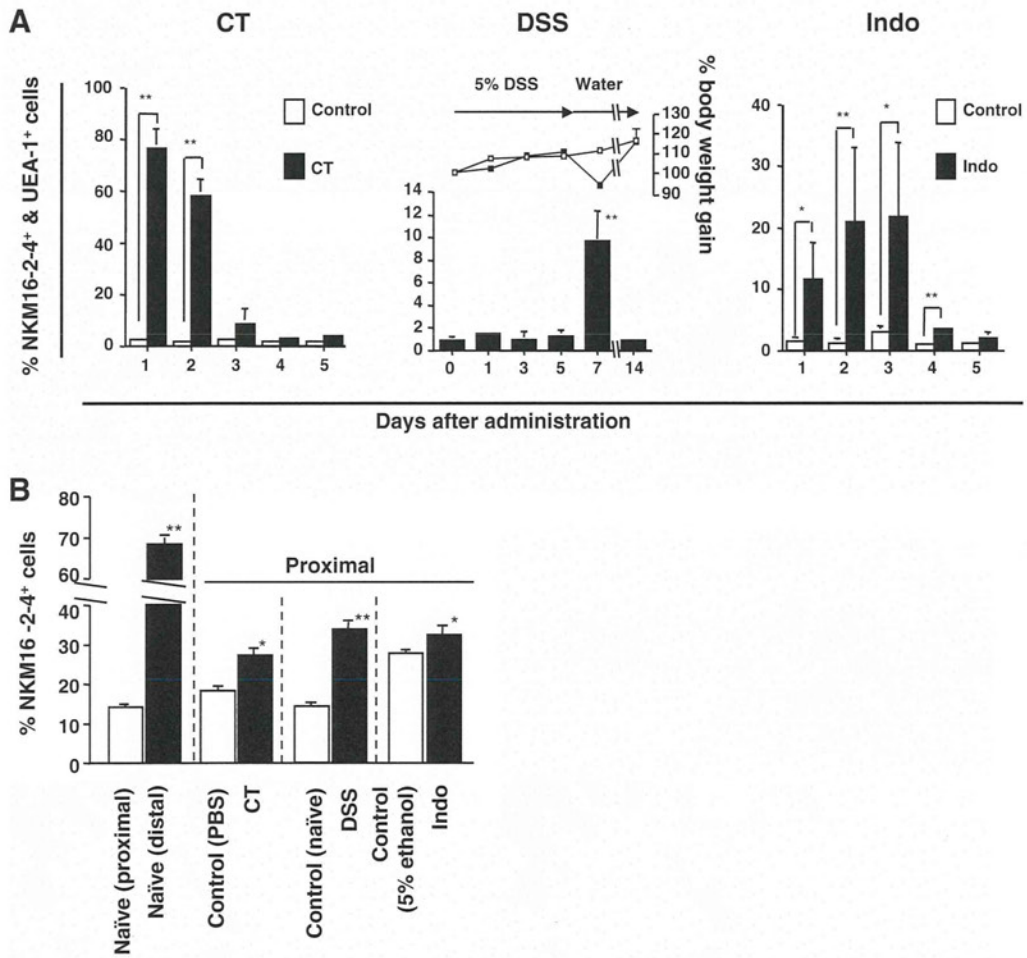

Fig. 1. The influence of IES on the induction of α(1,2)fucosylation in the small intestinal epithelia of BALB/c mice. (A) Daily analysis of the frequency of NKM 16-2-4$^+$/UEA-1$^+$ cells in the proximal villous epithelium of CT-, DSS- and indomethacin (Indo)-administered mice based on FCM. The ratio of NKM 16-2-4$^+$/UEA-1$^+$ cells was enumerated in cells, with 7-aminoactinomycin D$^+$ dead cells, CD45$^+$ leukocytes and small forward- and side-scattered lymphocytes gated out. The line graph in the middle panel shows the percentage body weight gain of control (open squares) and DSS-administered (filled squares) mice. Data are given as means ± SE (n = 3–7). Significant differences ($^*P < 0.05$, $^{**}P < 0.01$) were determined by t-test or Mann–Whitney's U test. (B) The proportions of NKM 16-2-4$^+$ cells in the PP domes based on histoplanimetrical analysis of CLSM images. Mice used were naïve, or were administered CT (day 1), DSS (day 7) or Indo (day 1). Data are given as means ± SE (n = 3, 19–76 domes). Significant differences ($^*P < 0.05$, $^{**}P < 0.01$) were determined by t-test.