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TLRs Regulate the Gatekeeping Functions of the Intestinal Follicle-Associated Epithelium

Sophie Chabot, Jessica S. Wagner, Stephanie Farrant, and Marian R. Neutra

Initiation of adaptive mucosal immunity occurs in organized mucosal lymphoid tissues such as Peyer’s patches of the small intestine. Mucosal lymphoid follicles are covered by a specialized follicle-associated epithelium (FAE) that contains M cells, which mediate uptake and transepithelial transport of luminal Ags. FAE cells also produce chemokines that attract Ag-presenting dendritic cells (DCs). TLRs link innate and adaptive immunity, but their possible role in regulating FAE functions is unknown. We show that TLR2 is expressed in both FAE and villus epithelium, but TLR2 activation by peptidoglycan or Pam3Cys injected into the intestinal lumen of mice resulted in receptor redistribution in the FAE only. TLR2 activation enhanced transepithelial transport of microparticles by M cells in a dose-dependent manner. Furthermore, TLR2 activation induced the matrix metalloproteinase-dependent migration of subepithelial DCs into the FAE, but not into villus epithelium of wild-type and TLR4-deficient mice. These responses were not observed in TLR2-deficient mice. Thus, the FAE of Peyer’s patches responds to TLR2 ligands in a manner that is distinct from the villus epithelium. Intraluminal LPS, a TLR4 ligand, also enhanced microparticle uptake by the FAE and induced DC migration into the FAE, suggesting that other TLRs may modulate FAE functions. We conclude that TLR-mediated signals regulate the gatekeeping functions of the FAE to promote Ag capture by DCs in organized mucosal lymphoid tissues. The Journal of Immunology, 2006, 176: 4275–4283.
CCL9 (MIP1\gamma), that are not expressed by villus epithelial cells and that attract DCs to the subepithelial dome (SED) region under the FAE (39–41). This presumably benefits the host by positioning DCs for efficient capture of the foreign materials transported by M cells. Whether TLRs are expressed on FAE enterocytes, and whether they play a role in the specialized functions of the FAE, has not been examined. This study was initiated to investigate the expression and localization of TLRs in epithelial cells of the FAE in mouse PP, including TLR2, -4, -5, and -9 that recognize pathogen-associated molecular patterns commonly present in enteric microorganisms and that are targets of mucosal vaccine adjuvants (42). Because the specialized phenotypes of epithelial cells and their interaction with DCs in PP have not been reproduced in vitro, we focused this study on PP in vivo. The distribution of epithelial TLRs was polarized in epithelial cells of the FAE. Injection of TLR2 ligands into the intestinal lumen in vivo had dramatic effects on TLR2 localization in FAE cells, but not in epithelia of villi or crypts. TLR2 ligands enhanced M cell transport of microparticles and induced DC migration into the FAE. Similar effects were observed using the TLR4 ligand LPS. Thus, the FAE has a unique response to TLR ligands, and TLR-mediated signals promote mucosal Ag sampling in organized mucosal lymphoid tissues. These findings are potentially important for understanding microbial pathogenesis in mucosal tissues and for the design of mucosal vaccines.

Materials and Methods

**Mice**

BALB/c, C57BL/6, C57BL/J, and C57BL/10ScNcr (TLR4-deficient) female mice 6–8 wk of age were obtained from Charles River Laboratories. C57BL/6-derived TLR2-deficient mice (originally produced by Dr. S. Akira, Osaka University, Osaka, Japan) were generously provided by Dr. D. Golenbock (University of Massachusetts, Worcester, MA). Mice were maintained in laminar flow cages and were free of specific microbial and viral pathogens as determined by plasma Ab screening. All animal procedures were performed in compliance with the guidelines for animal experimentation of Harvard Medical School, the Children’s Hospital Boston, and the National Institutes of Health.

**Ligated intestinal loops**

Mice were anesthetized by i.p. injection of avertin (tribromoethanol in t-amyl alcohol; 200 mg/kg animal weight), and a 3- to 5-cm segment of ileum containing a PP was ligated and injected as described previously (43). Loops were injected with 200 μl of PBS containing TLR agonists, which include *Staphylococcus aureus* PGN (Fluka; Sigma-Aldrich), PamCys (EMC), or ultrapure *Staphylococcus aureus* PGN (InvivoGen) at concentrations of 0.01, 0.1, or 1 mg/ml. Where indicated, TLR ligands were injected in combination with fluoresbrite plain yellow-green 0.2-μm microparticles (Polyscience), 2 μg/ml anti-TLR2 Abs (Santa Cruz Laboratories), 5 μg/ml GM6001 matrix metalloproteinase (MMP) inhibitor (Iomastat; Chemicon) or PBS alone. After 45 or 90 min, animals were sacrificed, and PP were excised, fixed, and labeled with fluorophore-conjugated secondary Abs do not recognize TLR2 in formaldehyde-fixed tissues, and unligated PP degenerates rapidly ex vivo. Therefore, to obtain an FAE sample or per standard villus sample of CD11c+ DCs within the FAE, a segment of FAE measuring 223 μm in length was demarcated, all CD11c+ cells located within the segment were counted, and DC counts in four FAE segments were totaled to obtain a standard FAE sample for each mouse. Data were expressed as the mean number of particles per standard FAE sample (n = 4 mice) ± the SDs. To quantitate relative numbers of intraepithelial CD11c+ DCs within the FAE, a segment of FAE measuring 223 μm in length was demarcated, all CD11c+ cells located within the segment were counted, and DC counts in four FAE segments were totaled to obtain a standard FAE sample for each mouse. To quantitate DCs within the villus epithelium of the same mice, separate villi sectioned longitudinally and lying within a 0.04-mm area of the PP sections were identified, and the total number of CD11c+ cells within the epithelium of four villi, a standard villus sample, was recorded for each mouse. Data were expressed as mean DCs per standard FAE sample or per standard villus sample ± the SDs. Statistical analyses were performed using Instat (GraphPad). Differences between groups were analyzed using the Student’s unpaired t test. A Bonferroni multiple comparison one-way ANOVA. Differences with *p* < 0.05; **p** < 0.01; and ***p*** < 0.001 were considered statistically significant. To analyze the difference between LPS-treated samples and respective PBS controls, an unpaired t test was performed.

**Results**

**TLR2 expression is polarized in intestinal FAE cells**

Expression of TLR2 in epithelia of unmanipulated animals was studied using whole-mount preparations of intact mouse PP and thin (3 μm) frozen tissue sections. Surface staining of the epithelium in formaldehyde-fixed whole mounts of BALB/c mouse PP with WGA allowed us to visualize the surface of the FAE on an individual lymphoid follicle surrounded by villi (Fig. 1a). Available anti-TLR2 Abs do not recognize TLR2 in formaldehyde-fixed tissues, and unfixed PP degenerates rapidly ex vivo. Therefore, to obtain an overview of TLR2 distribution on the surface of intact, fresh PP mucosa, rabbit anti-mouse TLR2 Abs or control rabbit IgG was injected into ligated segments of ileum in vivo, and 1 h later tissue was excised, fixed, and labeled with fluorophore-conjugated secondary Abs. The fixed tissue was treated with fluorophore-conjugated UEA-I to label apical surfaces of M cells and fluorophore-conjugated WGA to label all epithelial surfaces, and tissue was analyzed by confocal microscopy. A 1.5-μm stack of confocal planes containing WGA-labeled epithelial surfaces was selected to visualize the apical poles of epithelial cells including enterocyte brush borders, M cell surfaces, and apical endosomes. The WGA signal was subsequently subtracted...
FIGURE 1. TLR2 is expressed in mouse PP epithelial cells. a. and b. Intact, whole-mount preparation of mouse PP. a. Staining of the intact tissue with the lectin WGA and analysis by confocal microscopy shows the FAE of an individual follicle surrounded by villi. b. Anti-TLR2 Abs were applied in vivo and visualized on intact tissue at lower (top panels; scale bar, 30 μm) and higher magnification (bottom panels; scale bar, 10 μm). Anti-TLR2 (green) is detected in apical poles of cells in the FAE (left panels) but not in control FAE exposed to nonspecific rabbit IgG (right panels). M cell surfaces are stained with the lectin UEA-1 (red). The whole-mount preparations shown are representative of three mice. 
c. Tissue section of BALB/c mouse PP stained with anti-TLR2 (green) and UEA-1 (red). TLR2 is present at apical and basal poles of FAE cells. Nuclei are stained blue with DAPI (scale bar, 5 μm). d. FAE stained with anti-TLR2 (green) and WGA (red) shows that TLR2 is present below the brush border glycocalyx (scale bar, 5 μm). e. Negative control section stained with nonspecific rabbit IgG (green) and UEA-1 (red) (scale bar, 8 μm). f. Tissue section stained with anti-TLR2 (green) shows that TLR2 is confined to apical poles of villus and crypt epithelial cells (scale bar, 8 μm). Pictures shown (c–f) are representative of staining observed in BALB/c (n = 15) and C57BL/6 (n = 9) mice. g. TLR2 is not detected in the FAE of TLR2-deficient mice (scale bar, 8 μm). The dotted line indicates the basal side of the FAE. Staining shown is representative of all TLR2-deficient mice (n = 11).

From the confocal images shown, Anti-TLR2 Abs were present at apical poles of both FAE and villus epithelium (Fig. 1b). In the FAE, highest levels of anti-TLR2 staining occurred on FAE enterocytes, and lower levels were observed on some (but not all) UEA-1+ M cells (Fig. 1b). Tissues of normal BALB/c mice exposed to nonspecific rabbit IgG showed no significant label (Fig. 1b), and no staining was observed when anti-TLR2 was injected into ligated loops of TLR2-deficient mice (data not shown). Because labeling of the intact mucosal preparations in vivo presumably allowed uptake of TLR2-Ab complexes, the distribution of TLR2 was confirmed by application of anti-TLR2 Abs to frozen sections of unfixed PP tissue. This revealed TLR2 on or near the apical surfaces of FAE, villus, and crypt epithelial cells, including a subset of M cells (Fig. 1c and f). TLR2 was also present at the basal poles of FAE cells (Fig. 1c) but not at basal poles of villus or crypt epithelial cells (Fig. 1f). Double-staining with WGA, which labels the brush border glycocalyx located above the tips of epithelial microvilli, showed that TLR2 was localized below the glycocalyx, consistent with an apical membrane and/or apical endosome distribution (Fig. 1d). A similar distribution of TLR2 was seen in PP tissues of C57BL/6 mice (data not shown). Control tissue sections labeled with nonspecific rabbit IgG (Fig. 1e), and anti-TLR2 labeling of sections from TLR2-deficient mice (Fig. 1g) showed no staining, confirming the specificity of the anti-TLR2 Abs used.

Luminal TLR2 ligands induce the redistribution of TLR2 in cells of the FAE but not in villus or crypt epithelium

To test the effect of receptor ligation on the distribution of TLR2 in epithelial cells, PGN or Pam3Cys, ligands for TLR2, were injected into ligated loops containing a single PP in the mid-region of the small intestine, and PP tissues were harvested and frozen 90 min later. For most experiments, a high dose (1 mg/ml) of TLR2 ligands was used in an attempt to override possible effects of endogenous TLR2 ligands from commensal bacteria, but 0.01–0.1 mg/ml PGN produced similar effects on TLR2 distribution. Anti-TLR2 labeling of frozen tissue sections showed a reduction, but not loss, of TLR2 on villus epithelial surfaces (Fig. 2a). In the FAE exposed to PGN or Pam3Cys, UEA-1+ M cells appeared to contain higher levels of TLR2 intracellularly than in controls, whereas TLR2 was no longer detected in FAE enterocytes (Fig. 2b). TLR2+ cells were detected in the subepithelial region underneath the FAE only after exposure to PGN or Pam3Cys (Fig. 2b), and double-labeling with anti-CD11c Abs indicated that most of these cells were DCs (data not shown).

It has been proposed that interaction of TLR4 and TLR2 may be required for TLR2 signaling (44) because PGN-mediated responses in cultured cells were dependent on expression of both TLR2 and TLR4 (45). To determine whether PGN-mediated redistribution of TLR2 in...
the FAE in vivo was dependent on the presence of TLR4, PGN was injected into ligated intestinal loops in C57BL/10ScNcr TLR4-deficient and C57BL/10 control mice. PGN induced the same redistribution of TLR2 in FAE of both control C57BL/10 (TLR4+/H11001) mice and TLR4-deficient mice (data not shown), as observed in BALB/c mice (Fig. 2b), indicating that the response was not TLR4 dependent. The distribution of TLR4, -5, and -9 in PP of normal BALB/c and C57BL/6 mice was then examined by immunostaining of frozen tissue sections. In both mouse strains, Abs recognizing the N terminus of TLR4 revealed low or undetectable levels of TLR4 at apical surfaces of FAE and villus epithelial cells (Fig. 3), and higher levels at apical surfaces of crypt cells (data not shown) as reported previously (26). Labeling was TLR4 specific, because the Abs failed to label tissues from TLR4-deficient mice (data not shown). Abs specific for the TLR4/MD2 signaling complex labeled subepithelial CD11c+ DCs, but not the FAE (Fig. 3). Although mice deficient in TLR5 and TLR9 were not available to verify the specificity of the Abs used, anti-TLR5 labeled apical poles and supranuclear structures in FAE as well as villus (Fig. 3) and crypt epithelial cells (data not shown). The supranuclear structures were also labeled with the lectin *Helix pomatia* agglutinin (Fig. 3, inset), a marker for nascent complex carbohydrates in the Golgi complex (46). TLR9 was detected at both apical and basal poles of the FAE (Fig. 3) and crypt epithelial cells (data not shown), but only at the apical poles of villus epithelial cells (Fig. 3). The distribution of TLR2, -5, and -9 in TLR4-deficient mice was indistinguishable from that of control mice. Furthermore, the distribution of TLR4, -5, and -9 in FAE or villus epithelium was not affected by treatment with luminal PGN in either wild-type or TLR4-deficient mice (data not shown), suggesting that the observed redistribution of TLR2 in response to TLR2 ligands was due to specific TLR2-ligand interaction.

**TLR2 ligands enhance FAE transport of microparticles**

We then sought to determine whether TLR2 ligands might have an effect on FAE functions such as M cell transport. M cells in rabbits (47) and mice (48) are known to endocytose and transport fluorescent latex microspheres across the FAE. Ligated intestinal loops of control BALB/c, C57BL/6, and TLR2-deficient mice were therefore injected with 200-nm fluorescent latex microspheres, and tissue was harvested, rinsed, and fixed 90 min later. Microspheres on the surface of the FAE, within the epithelium and in the underlying tissue, were counted in standard areas on sections of PP follicle domes as described in Materials and Methods. In untreated or PBS-injected wild-type and TLR2-deficient mice, the mean numbers of microspheres per standard area were similar (Fig. 4, a and b). When 1 mg/ml PGN was injected along with microspheres, however, the number of microspheres within and under the FAE...
was significantly enhanced in wild-type BALB/c mice (data not shown), wild-type C57BL/6 mice, wild-type C57BL/10 mice, and TLR4-deficient mice, but not in TLR2-deficient mice (Fig. 4b). The effect of PGN on microsphere uptake could be detected after 45 min, and the effect was dose dependent (Fig. 4c). UEA-1 labeling of tissue sections from BALB/c mice given injections with 100 μg/ml PGN showed colocalization of microspheres with UEA-1+ M cells, as expected (Fig. 4d). These results suggest that luminal PGN modulates the transport functions of M cells in the FAE through a TLR2-mediated mechanism.

FIGURE 4. PGN enhances uptake of fluorescent latex microspheres by the FAE in a TLR2-dependent manner. a, Ligated intestinal loops were injected with 200-nm fluorescent latex microspheres (beads) and either 1 mg/ml PGN or PBS (control) in wild-type mice (above) and TLR2-deficient mice (below). PGN increased uptake of fluorescent latex microspheres across the FAE in wild-type but not in TLR2-deficient mice. b and c, Relative number of fluorescent latex microspheres associated with the FAE and underlying tissue. Bars represent the mean number of microspheres per standard FAE sample ± SD. A standard FAE sample was obtained from each of four mice in all groups (n = 4), except control C57BL/10 mice (n = 2). Each standard FAE sample represents the total number of microspheres counted in standard areas of FAE sections from four separate follicles in a single PP as described in Materials and Methods. b, PGN- (1 mg/ml) enhanced microsphere uptake in wild-type C57BL/6 (TLR2 wild-type; wt), wild-type C57BL/10 (TLR4 wt), and TLR4-deficient C57BL/10ScNcr (TLR4−/−) mice, but not in TLR2-deficient C57BL/6 (TLR2−/−) mice. Significance between PGN and respective PBS control are indicated (**, p < 0.01; ***, p < 0.001). c, Increased uptake of microspheres induced by PGN was significant at 45 and 90 min and was dose dependent. d, A section of FAE from a BALB/c mouse exposed to PGN (100 μg/ml) and fluorescent latex microspheres (green) stained with UEA-1 (red), a marker for M cells, confirms that uptake of microspheres was mediated by M cells. The picture shown is representative of staining observed in all PGN-exposed tissues of BALB/c mice (n = 15).
TLR2 ligands induce migration of subepithelial DCs into the FAE

Another important function of the FAE is to attract DCs to the SED region, presumably to capture transported Ags (39–41, 49). To test the possibility that PGN affected the movements of PP DCs, the distribution of DCs in PP exposed to PGN for 90 min was compared with that of PBS-injected controls by labeling frozen tissue sections with anti-CD11c Abs. To visualize the boundary between the epithelium and connective tissue, basement membranes were stained with anti-laminin Abs. In normal BALB/c mice and PBS-injected controls, many CD11c+ DCs were present in the SED region under the FAE, and a few DCs were detected within the FAE (Fig. 5a) as reported by others (39, 49). In PP exposed to luminal PGN at concentrations of 0.01, 0.1, or 1 mg/ml, a significant increase in the numbers of CD11c+ DCs in the FAE was observed in BALB/c (Fig. 5a), wild-type C57BL/6, and TLR4-deficient mice, but not in TLR2-deficient mice (Fig. 5c). In contrast, PGN had no effect on the number of DCs in the villus epithelium of any mouse strain (Fig. 5, b and c). Similar results were observed after injection of Pam3Cys (data not shown). Finally, intraluminal injection of GM6001 (5 μg/ml), a broad-range inhibitor of MMPs, significantly inhibited PGN-induced migration of DC into the FAE (Fig. 5d). However, GM6001 treatment did not prevent the redistribution of TLR2 in the FAE or the enhanced uptake of microspheres mediated by PGN (data not shown). Taken together, these results suggest that DCs in the SED region of PP

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** PGN induces rapid migration of DCs into the FAE, but not into villus epithelium. Tissue sections of BALB/c mouse PP from ligated intestinal loops injected with 1 mg/ml PGN or PBS (control) were stained for CD11c (green), laminin (red), and nuclei (blue). a and b, Arrows indicate DCs in the epithelium after 90 min of PGN exposure. Similar results were obtained using lower doses of PGN (10–100 μg/ml). PGN induced migration of DCs into the FAE (a) but not into villus epithelium (b). Pictures shown (a and b) are representative of staining observed in BALB/c mice (PBS control, n = 15; PGN-treated, n = 11). c and d, Quantitation of CD11c+ DCs in FAE or villus epithelium, as described in Materials and Methods. Values shown are expressed as the mean number of DCs per standard FAE sample ± SD (n = 4 mice). Each standard FAE sample represents the total number of DCs counted in four standard lengths of FAE from four separate follicles in a single PP from one mouse. c, PGN (1 mg/ml) significantly increased DCs in the FAE of both wild-type strains (wt) and TLR4-deficient (−/−) mice, but not in TLR2-deficient (−/−) mice. **p < 0.05; ***p < 0.001; and ****p < 0.0001, PGN treated compared with respective PBS controls. d, A broad-range MMP inhibitor, GM6001, prevented the PGN-induced migration of DCs into the FAE of BALB/c mice. ****p < 0.001, MMP inhibitor/PGN treated compared with PGN alone.
respond to TLR2-mediated signals, and that TLR2-mediated DC migration into the FAE is dependent on the activation of MMPs.

LPS, a TLR4 ligand, also modulates gatekeeping functions of the FAE

To determine whether other TLR ligands can affect M cell transport and/or DC migration in the FAE, LPS (100 μg/ml) was injected into ligated loops of BALB/c mice, and the effects on microparticle uptake and DC migration were evaluated after 90 min. LPS significantly enhanced the uptake of microparticles by the FAE (Fig. 6a) and also induced migration of DCs into the FAE (Fig. 6b). These results suggest that TLRs other than TLR2 can modulate FAE functions.

Discussion

The FAE associated with organized mucosal lymphoid tissues represents only a small fraction of the intestinal surface area, but it plays a crucial role in the initiation of mucosal immune responses against foreign Ags and pathogens. M cells in the FAE transport samples of the luminal content across the epithelial barrier (31) and FAE enterocytes release chemokines that attract Ag-presenting DCs to the subepithelial region (39–41), where these samples can be efficiently captured. We have now shown that the distribution of TLR2 in epithelial cells of the FAE, including M cells, differs from that of villus and crypt epithelial cells. Injection of TLR2 ligands into the intestinal lumen induced responses in the FAE that were not observed in adjacent villi. These included TLR2 redistribution, enhanced uptake of microparticles by M cells, and induced migration of DCs into the epithelium.

In the intestinal villi and crypts of the normal BALB/c and C57BL/6 mice used in this study, the TLRs detected by immunocytochemistry (TLR2, -4, -5, and -9) were located at the apical poles of epithelial cells. This is consistent with previous reports of the polarized apical distribution of TLR3 (10), TLR4/MD2 (26), and TLR5 (3) in normal mouse intestinal epithelium. We observed that TLR2, -4, -5 and -9 are also present in the FAE of mouse PP, and that TLR4 and -5 show the same distribution in FAE cells as in villus epithelial cells. In contrast, TLR2 and TLR9 were observed at both apical and basal poles of cells in the FAE, but at apical poles only in villus and crypt epithelial cells. Apical TLR2, -5, and -9 were located just below the lectin-labeled glycocalyx, suggesting that these receptors may be present in microvillous membranes, apical endosomes, or both. Apical membrane components are known to recycle between microvillus membrane and apical endosomes (50), which is consistent with the availability of TLRs at intestinal cell surfaces as previously reported for TLR2 and TLR4 (8). The presence of basolateral TLR2 and TLR9 on FAE but not on villus epithelium may reflect the contrasting functions of these distinct epithelia. The villus epithelium is designed to prevent the entry of Ags and microorganisms, and apical TLRs on villus enterocytes may participate in the “sensing” of luminal microorganisms (1) that is essential for maintaining epithelial homeostasis (28). In contrast, the apical and basal distribution of TLR2 and TLR9 on the FAE may reflect the presence of microorganisms and their components on both sides of the epithelial barrier as a result of constitutive transepithelial transport by M cells. For the recognition of PGN in particular, other receptors such as peptidoglycan recognition protein (PGRP)-L and PGRP-S, which are exclusively expressed on the FAE cells, may also be involved (51).

TLR2 ligands injected into the intestinal lumen induced a dramatic loss of immunoreactive apical and basolateral TLR2 in FAE enterocytes, whereas apical TLR2 in the villus epithelium was decreased but not lost. Others have reported that low levels of TLRs are correlated with unresponsiveness to TLR ligands, including PGN, and with the relatively high levels of inhibitory Tollip mRNA recovered by laser capture microdissection from human villus enterocytes (6). The rapid loss of TLR2 from FAE enterocytes could reflect endocytosis that may be required for TLR-mediated signaling (8, 52, 53) and for degradation of receptors resulting in the termination of TLR-mediated responses (54, 55). Further studies following the fate of TLR2 in PP early after exposure to TLR2 ligands will be required to elucidate the fate of TLR2-ligand complexes. Nevertheless, the observed loss of TLR2 suggests that FAE enterocytes play a role in TLR2-mediated responses of the FAE.

In contrast to FAE enterocytes, M cells in PP remained strongly TLR2-positive after exposure to high doses of PGN. However, the distribution of TLR2 in these cells appeared to shift from predominantly apical, to apical, intracellular, and basolateral, consistent with possible entry of TLR2-ligand complexes into the well-documented transepithelial vesicular transport pathway of these cells (31). The persistence of TLR2 in M cells is consistent with the fact that materials taken up by these cells are generally not directed to lysosomes and are not degraded; rather, M cell endocytic vesicles deliver their content directly to the basolateral cell surface (31). The fact that immunoreactive TLR2 was readily detected intracellularly in PGN-exposed M cells suggests that PGN did not interfere with Ab binding sites on TLR2, and that the loss of TLR2 observed in FAE enterocytes was not due simply to blocking of Ab binding sites by PGN. The uptake of TLR2 by M cells suggests that this receptor may carry endocytosed ligand and mediate the delivery of microbial components such as PGN, or intact Gram-positive bacteria displaying TLR2 ligands, across the epithelium into the PP. Such a transport function has been suggested for the alternate PGN receptor PGRP-S expressed on UEA⁺M cells in mice (51).

TLR2 ligation also had a rapid effect on M cell endocytic and transcytotic activity, significantly increasing the uptake and transport of latex microspheres. It is important to note that enhanced

![FIGURE 6](http://www.jimmunol.org/) LPS modulates gatekeeping functions of the FAE. a, Ultrapure *E. coli* LPS (100 μg/ml) injected into ligated intestinal loops for 90 min enhanced the rate of transepithelial transport of microparticles by the FAE. The numbers of fluorescent latex microspheres associated with the FAE and underlying tissue in BALB/c mice (*n* = 4) were counted as described in Materials and Methods. b, LPS also induced DC migration into the FAE of BALB/c mice (*n* = 4). For both graphs, values are expressed as the mean number of beads or DCs per standard FAE sample ± SD. Each standard FAE sample included the total number of beads or DCs counted in standard areas of FAE in sections from four separate follicles in a single PP. *, *p* < 0.05 compared with PBS control.
transcytosis in response to TLR2 ligands was TLR2 dependent and was not mediated by PGRP, because it occurred in wild-type and TLR4-deficient mice, but not in TLR2-deficient mice. The fact that LPS had a similar effect on microparticle transport suggests that M cells may respond to many types of bacteria through multiple TLRs. It is known that although M cell transport is a constitutive process, these cells are capable of increasing the rate of transcytosis over the short term. In a recent study, the rate of Ag uptake into PP was enhanced by exposure to Streptococcus pneumoniae, and this did not require formation of additional M cells (56). The molecular mechanisms regulating the rate of transepithelial transport by M cells are unknown, but this study provides evidence that TLRs may be involved.

TLR2 ligands also induced the rapid migration of subepithelial DCs into the FAE but not into villus or crypt epithelium. This phenomenon was dependent on TLR2 because it was observed in wild-type or TLR4-deficient, but not TLR2-deficient mice. Although it was not possible to determine the origin of the intraepithelial DCs, they appeared within 45 min after TLR2 ligand stimulation, and it seems reasonable to assume that they came from the large congregation of DCs that normally occupies the SED region (39, 49). M cell transport of ligands into the SED region can occur in <45 min (31), and thus the PGN-induced migration of DCs observed could have involved TLR2 on epithelial cells, subepithelial DCs, or both. One possibility is that TLR2 signaling attracted DCs through release of epithelial chemokines. Release of CC chemokines MIP1γ and MIP3α by the FAE can attract CCR6+ and perhaps CCR1+ DCs to the SED region (39–41, 57), and fractalkine (CX3CL1) produced by the epithelium of small intestinal villi (58) may activate CX3CR1 on DCs to stimulate extension of dendrites into the epithelium (59). It is known that mRNA and protein expression of MIP3α by epithelial cells can be induced by TLR stimulation (11, 57, 60). However, in our system, migration of DCs into the FAE was observed 45 min after exposure to PGN, a period insufficient for de novo gene up-regulation to occur. TLR2 ligation may have resulted in release of preformed DC-attracting chemokines from the epithelium. Membrane-associated fractalkine can be rapidly released from cell membranes through cleavage by MMPs (58, 61, 62). The observation that inhibition of MMP activity significantly reduced the migration of DCs into the FAE in our experiments is consistent with the possibility that TLR2 ligation resulted in MMP activation in epithelial cells. Alternatively, TLR2 ligands transcytosed by M cells could have interacted directly with TLR2 on subepithelial DCs, and MMP activation by DCs could have facilitated their movement through the extracellular matrix and into the epithelium (63). The fact that enhancement of M cell transport by PGN was unaffected by MMP inhibition indicates that migration of DCs into the FAE was not required for the M cell effect.

Although the focus of the present study was TLR2, it should be emphasized that signaling through other TLRs may also modulate FAE functions. Indeed, we observed that injection of LPS, a ligand of TLR4, into ligated loops also enhanced microparticle uptake by epithelial DCs through release of epithelial chemokines. Membrane-associated fractalkine (CX3CL1) produced by the epithelium of small intestinal villi (58) may activate CX3CR1 on DCs to stimulate extension of dendrites into the epithelium (59). It is known that mRNA and protein expression of MIP3α by epithelial cells can be induced by TLR stimulation (11, 57, 60). However, in our system, migration of DCs into the FAE was observed 45 min after exposure to PGN, a period insufficient for de novo gene up-regulation to occur. TLR2 ligation may have resulted in release of preformed DC-attracting chemokines from the epithelium. Membrane-associated fractalkine can be rapidly released from cell membranes through cleavage by MMPs (58, 61, 62). The observation that inhibition of MMP activity significantly reduced the migration of DCs into the FAE in our experiments is consistent with the possibility that TLR2 ligation resulted in MMP activation in epithelial cells. Alternatively, TLR2 ligands transcytosed by M cells could have interacted directly with TLR2 on subepithelial DCs, and MMP activation by DCs could have facilitated their movement through the extracellular matrix and into the epithelium (63). The fact that enhancement of M cell transport by PGN was unaffected by MMP inhibition indicates that migration of DCs into the FAE was not required for the M cell effect.

Epithelial-DC interactions at mucosal surfaces are thought to provide a crucial link between innate and adaptive mucosal immunity (64–66). The TLR2-mediated effects on M cell transport and DC migration that we observed in vivo would be expected to result in enhanced Ag capture in organized mucosal lymphoid tissues, but not in the general mucosa. The TLR2-induced migration of DCs into the FAE but not into villus epithelium that we observed is consistent with evidence that DCs associated with the FAE have a distinct phenotype from those of the lamina propria (39), and with other evidence that subepithelial DC functions are influenced by epithelial signals (39–41, 48). CROSSTALK between villus epithelial cells and DCs may promote intestinal immune homeostasis (65), whereas crosstalk between FAE cells and DCs may influence the nature of immune responses initiated in mucosal-organized lymphoid tissues (67). Our results support the hypothesis that a unique crosstalk between epithelial cells of the FAE and DCs occurs in mucosal-organized lymphoid tissues, and that TLRs participate in this important interaction.

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Disclosures

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