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Innate immune responses of human tracheal epithelium to *Pseudomonas aeruginosa* flagellin, TNF-α, and IL-1β

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Tseng, Jill, Jiun Do, Jonathan H. Widdicombe, and Terry E. Machen. Innate immune responses of human tracheal epithelium to *Pseudomonas aeruginosa* flagellin, TNF-α, and IL-1β. *Am J Physiol Cell Physiol* 290: C678–C690, 2006. First published October 26, 2005; doi:10.1152/ajpcell.00166.2005.—We measured innate immune responses by primary human tracheal epithelial (HTE) cells grown as confluent, pseudostratified layers during exposure to inflammatory activators on apical vs. basolateral surfaces. Apical *Pseudomonas aeruginosa* strain PAK (but not flagellin mutant PAK·fliC), flagellin, and flagellin + PAK·fliC activated NF-κB and IL-8 expression and secretion. In contrast, HTE cells were insensitive to LPS compared to flagellin. Flagellin activated NF-κB in columnar but not basal cells. IL-1β + TNF-α elicited responses similar to those of flagellin. Basolateral flagellin or IL-1β + TNF-α caused 1.5- to 4-fold larger responses, consistent with the fact that NF-κB activation occurred in both columnar and basal cells. MyD88 (toll receptor-associated adapter), IL-1 receptor (IL1R)1, and TNF-α receptor (TNFR)1 were expressed in columnar and basal cells. ZO-1 was localized to tight junctions of columnar cells but not to basal cells. We infer the following. *1*) Flagellin is necessary and sufficient to trigger inflammatory responses in columnar cells during accumulation of *P. aeruginosa* in the airway surface liquid (ASL); columnar cells express toll-like receptor 5 and MyD88, often associated with flagellin-activated cell signaling. *2*) IL-1β + TNF-α in the ASL also activate columnar cells, and these cells also express IL1R1 and TNFR1. *3*) Apical flagellin, IL-1β, and TNF-α do not activate basal cells because tight junctions between columnar cells prevent access from the apical surface to the basal cells. *4*) Exposure of basolateral surfaces to inflammatory activators elicits larger responses because both columnar and basal cells are activated, likely because both cell types express receptors for flagellin, IL-1β, and TNF-α.

toll-like receptor; nuclear factor-κB; interleukin-8; tumor necrosis factor; interleukin-1

UNDER NORMAL CONDITIONS, the lung airways remain largely free of pathogens. However, when bacteria accumulate, the epithelial cells lining the airways become activated to express many different genes (9, 17), including inflammatory cytokines like IL-1β, IL-6, IL-8, and TNF-α (10, 13, 19), which attract neutrophils from the blood vessels into the airways. This innate immune defense response is exaggerated in the disease cystic fibrosis (CF): large numbers of *Pseudomonas aeruginosa* accumulate in the airways, and these bacteria trigger a massive innate immune response.

Bacterial flagellin appears to be important in triggering the innate immune response in airways. Fla$^-$ mutants are much less virulent in a mouse model of pneumonia (6), flagella cause the upregulation of matrilysin in airway epithelial cells (characteristic of the innate immune response) (16), and *P. aeruginosa* fliC (flagellin structural gene) is required to activate NF-κB and increase the expression and secretion of IL-1β, IL-6, IL-8, and TNF-α by CF15 cells (10). However, other bacterial products [e.g., LPS (3, 15), pili (5)] may also stimulate the innate host response of airway epithelial cells, so the specific role of flagellin in the early phases of infection remains an open question. Toll-like receptor (TLR)5 is involved in the response of many cells to flagellin (9, 27), and the specific regions of TLR5 involved in binding flagellin monomers have been identified (25).

A major area of controversy has been the apical vs. basolateral responsiveness of airway and other epithelia to bacteria or purified flagellin and the identity and polarization of specific receptors involved in these responses in epithelial cells. For intestinal T84 cells, basolateral flagellin elicited secretion of IL-8, whereas apical treatment had no effect, and TLR5 appeared from antibody localization to be present primarily on the basolateral surface (7). However, recent studies showed flagellin-dependent responses and TLR5 to be present in the apical membrane of ex vivo intestinal epithelium (2). Our studies (10) of nasal CF15 cells found that flagellin-dependent responses were likely mediated through receptors on the basolateral surfaces of the cells. In the cornea, TLR5 appeared also to be located primarily on basolateral membranes (31) of basal cells. In contrast, other work has shown that airway epithelial cell lines and primary cells respond to apical flagellin, and these responses appeared to be mediated during an early phase through flagellin interacting with TLR2 and gangliotetraosylceramide (asialoGM1), which was proposed to serve as a coreceptor in the activation of Ca^{2+} signaling in the cells, whereas during later responses (after 4 h) flagellin appeared also to interact with TLR5 mobilized from an internal pool to the apical membrane (Ref. 26; also see Refs. 17, 21). Activation of intestinal Caco2 cells by flagellin may also involve a critical role for a glycosphingolipid similar to GM1 (18). It has also been proposed that there is prominent activation of innate immune responses by *P. aeruginosa* acting intracellularly (12).

There were three major goals of the present work. First, we wanted to determine the role of flagellin in *P. aeruginosa* activation of innate immune responses (activation of NF-κB and expression and secretion of IL-8) of primary cultures of polarized tracheal airway pseudostratified layers containing both columnar (ciliated and secretory) and basal epithelial cells, similar to the situation in vivo. We studied surface

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mucosa compared with glandular mucosa because this is the region that would most likely be contacted first by accumulating bacteria in *P. aeruginosa* infections. We determined responses to *P. aeruginosa* strain PAK, an isogenic mutant that does not express flagellin structural gene (PAKΔfliC), and *P. aeruginosa* flagellin. We also tested the effects of LPS to determine the relative sensitivity of the airway epithelia to LPS compared with flagellin. Results from all of these experiments showed that flagellin alone was sufficient to activate the innate immune response in airway epithelial cells.

Second, we compared the effects of apical vs. basolateral flagellin or TNF-α + IL-1β in activation of this innate immune response to determine whether bacterial activation of airway epithelial cells occurs through direct activation of apical receptors (26) or occurs indirectly through bacterial products activating macrophages, which release proinflammatory cytokines to activate further proinflammatory reactions in epithelial cells (12).

Third, we wanted to determine whether all or only specific cells in the layer respond to apical vs. basolateral *P. aeruginosa* flagellin and the proinflammatory cytokines to help clarify the early events in the epithelial host response to *P. aeruginosa*. Immunofluorescence and confocal microscopy were used to determine which cells responded to these activators. We used antibodies to MyD88, an adapter associated with toll receptors (27), TNF-α receptor (TNFR)1, and IL-1β receptor (IL1R)1 to determine whether these receptors were present in columnar cells and also basal cells. ZO-1 staining was used to identify the tight junctional regions of the cells. We also used antibodies to NF-κB p65 to determine which cells had been activated during apical or basolateral treatments with flagellin or TNF-α + IL-1β by identifying cells in which NF-κB had migrated from the cytosol to the nucleus.

MATERIALS AND METHODS

Reagents. P. aeruginosa flagellin (10^{-3} g/ml in solution containing 10 mM phosphate buffer pH 7.4, 140 mM NaCl, and 3 mM KCl; Inotek, Beverly, MA) was stored at −20°C and then diluted from the stock into the incubation medium at the indicated concentrations. As described by Inotek, recombinant flagellin is expressed with tags in *Escherichia coli* and purified to >95% homogeneity by SDS-PAGE. Recombinant human IL-1β and TNF-α (R&D Systems, Minneapolis, MN) were used at 50 ng/ml and 100 ng/ml, respectively. Unless otherwise specified, other reagents and chemicals were obtained from Sigma (St. Louis, MO). LPS from *P. aeruginosa* serotype 10 (Sigma; purified by gel-filtration chromatography) was dissolved as directed in H_2O at 10^{-3} g/ml and diluted to 5×10^{-8} g/ml in medium for experiments.

Pseudomonas aeruginosa. P. aeruginosa strain PAK was grown overnight in Luria-Bertani medium at 37°C with vigorous aeration. An isogenic mutant of *P. aeruginosa* PAK lacking flagellin (PAKΔfliC) obtained from S. Lory (Harvard Medical School, Boston, MA) was engineered by in-frame deletion of the *fliC* gene from the chromosome as described previously (10). These bacteria were handled in the same manner as the parent strain; all strains grew with similar rates. Before experiments, bacteria were washed three times with PBS and resuspended in assay medium [human tracheal epithelial (HTE) cell medium minus antibiotics and supplements] at a concentration of 10^8 colony-forming units/ml in a volume of 0.5 ml. The apical surface was washed once with the assay medium, followed by replacement with 0.5 ml of solution + bacteria on the apical surface of the HTE [i.e., ~40 multiplicity of infection (MOI)].

HTE monolayer cultures and protocols. Tracheal epithelial cells were obtained by protease digestion postmortem and then grown and passaged once, as described previously (10, 20, 26). These P1 ("first passage") cells were suspended in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM-F-12) containing 5% FBS and seeded at 5×10^5 cells/cm^2 on 1.13-cm^2 Transwell polycarbonate inserts (no. 3401; Costar, Corning, NY) in the bottom of 24-well plates. Growth surfaces were coated with human placental collagen. The day after being plated, the DMEM-F12 over the filters was replaced with Gray's medium, a medium whose composition is fully described elsewhere (22, 28–30). Once the cells had attained confluence, they were grown with an air-liquid interface (ALI; i.e., medium was only added to the outside of the insert, the cell's basolateral surface). Cells grown at the ALI and with basolateral Gray's medium develop a pseudostratified phenotype with both columnar (ciliated and nonciliated, secretory-like) cells and basal cells. Previous experiments showed that these monolayers contain epithelial cells with no obvious contamination from myeloid cells (30).

Transepithelial electrical resistance (R_T) was measured to ensure confluence of the filters. During this procedure we added 500 μl of PBS to the mucosal surface and then used a "chopstick" voltmeter (Millicell ERS; Millipore Products, Bedford, MA). The measurements took 1 min, after which the PBS was immediately removed. Experiments were performed 2–4 wk after plating (>10 days after attaining confluence). These cultures attained minimum R_T of >100 Ω·cm^2 and maintained an ALI or were discarded. The usual resistance of monolayers was R_T = 400–500 Ω·cm^2. The maintenance of the ALI and the different responses of the epithelial layers to flagellin, IL-1β, and TNF-α + IL-1β showed that the tight junctions were intact in all the epithelia used for this study.

HTE cells were left untreated or treated with *P. aeruginosa*, flagellin, or IL-1β and/or TNF-α on the apical surface for 4 h. These experiments tested the ability of HTE monolayers to respond to these bacteria and products and inflammatory cytokines. Similar measurements were performed on HTE monolayers treated with either flagellin or IL-1β and/or TNF-α added to the apical or basolateral sides of the monolayers. These experiments made direct comparisons of the apical vs. basolateral responses to flagellin or inflammatory cytokines. It was not possible to add bacteria to the basolateral solution because the HTE cells had to be grown on collagen-coated filters, and it seemed unlikely that the bacteria would be able to gain access to the HTE cells from the serosal solution across this barrier. In some experiments, samples were taken from the apical and/or basolateral solution for measurement of IL-8 secretion by ELISA. After these different treatments the cells were washed three times and then processed for either quantitative PCR (QPCR) or luciferase measurements or immunofluorescence as described below.

ELISA of IL-8 secretion. Samples were routinely collected from the basolateral chamber of cells grown on filters, and in some experiments also from the apical chamber. Samples from control or treated cells were collected, cleared of any bacteria or cellular debris by centrifugation (1 min, 10,000 *g*), stored at −20°C until use, and then thawed, diluted to 1:100 or 1:200 in 100 μl of assay diluent (BD Pharmingen, San Diego, CA), run in triplicate per the manufacturer's protocol (OptEIA Human IL-8 Set, BD Pharmingen), and read at 450 nm with an EL_X808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT). Averages of the triplicates are reported here.

QPCR to determine IL-8 and 18S gene expression. HTE cells were rinsed of adherent bacteria or other treatments by washing three times with PBS. RNA and cDNA were harvested and generated with a Cells-to-cDNA II kit (Ambion, Austin, TX) per the manufacturer's protocol. QPCR reactions were assembled in a 50-μl volume containing 1 μM forward primer, 1 μM primers, 200 μM dNTP with dUTP, 2.5 U AmpliTaqGold, 1× SYBR Green PCR buffer, and 300 nM $MgCl_2$. Reactions were amplified in a thermal cycler (GeneAmp5700, Perkin-Elmer, Wellesley, MA) with the following parameters: 30 min at 48°C, 10 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles.

The following primer sequences were used: 5′-ATG ACT TCC AAG CTG GCC GTG GCT (IL-8 forward), 5′-TCT CAG CCC TCT TCA AAA ACT TCT C (IL-8 reverse), 5′-TCA AGA AGG TGG TGA AGC AG (18S forward), and 5′-TCG CTG TTG AAG TCA GAG GA (18S reverse). Quantitation was calculated by comparing the threshold cycle number of each reaction to the standard curve generated from samples that contained serial dilutions of DNA template. Each sample was performed in triplicate to obtain an average, which was then normalized by expressing as a ratio of IL-8 over 18S quantity for matching samples.

NF-κB-regulated luciferase. A recombinant adenoviral vector expressing a luciferase reporter gene driven by NF-κB transcriptional activation (adv-NF-κB-luc; from University of Iowa Vector Core) was used for functional studies as described previously (23). This vector contained the luciferase gene driven by four tandem copies of the NF-κB consensus sequence. Recombinant adenoviral stocks were generated as previously described (23) and were stored in 10 mM Tris with 20% glycerol at −80°C. The virus was added to the basolateral surface of HTE cell monolayers at 100 MOI and returned to the incubator for 48 h, when experiments were performed. Control experiments using lacZ-expressing adenovirus showed that this concentration of virus was sufficient to infect 80–100% of cultured airway epithelial cells grown on filters. After experimental procedures, HTE cells were washed and processed with the luciferase assay system with reporter lysis buffer (Promega, Madison, WI) to measure NF-κB-mediated transcriptional induction according to the manufacturer's protocol. Luciferase activities (in relative light units) were measured in triplicate for each sample and normalized to the protein concentration (Bradford assay). These averages were then expressed relative to the average control value (absence of bacteria), which was set equal to 1.0.

Immunofluorescence. For NF-κB localization to the cytosol and nucleus, HTE cells were treated with *P. aeruginosa* or mutants, flagellin, or TNF-α + IL-1β for 1 h and then rinsed three times with PBS, fixed by treatment for 15 min with −20°C methanol, rinsed with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 15 min. After blocking with 1% BSA-PBS for 20 min, cells were incubated for 1 h at room temperature with an anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Alexa 546-coupled goat secondary antibody. In most experiments we also included 5 μM 4′,6′-diamidino-2-phenylindole (DAPI; Molecular Probes) to stain nuclei. Images were obtained with a laser scanning confocal microscope (Zeiss, Oberkochen, Germany) with a ×63 oil objective (numerical aperture = 1.4). Enhancements were performed with Volocity LE (Improvision, Lexington, MA) and/or Photoshop 7 (Adobe, San Jose, CA).

For quantitation of the number of surface and basal cells in which NF-κB had migrated to the nucleus for a particular experimental condition, 4–11 random fields were selected and counted for total cell number (total number of nuclei) and for cells with active (nuclear) NF-κB. Columnar cells were distinguished from the basal cells growing near the filter by focusing on nuclei in the top nuclear layer of the HTE monolayer, within 5–10 μm of the cell surface. Nuclei in the basal cells were then identified by focusing on the next layer more basal to those in the superficial layer, usually 5–15 μm more basal. Cells with activated, nuclear NF-κB were identified as those in which there was clear accumulation of red NF-κB staining throughout the nucleus.

Separate experiments were performed to identify ciliated cells and basal cells expressing MyD88, IL1R1, or TNFR1. Apical tight junction regions of the cells were identified from ZO-1 staining. Primary cell monolayers were left untreated or treated with apical flagellin or IL-1β + TNF-α for 1 h and then fixed, permeabilized, and treated with antibodies as above. Anti-centrin and anti-ZO-1 antibodies and Alexa 488 secondary antibodies were used to identify ciliated cells and tight junction regions of columnar cells, respectively. Anti-MyD88 or anti-TNFR1 antibodies were used with secondary Alexa 633 antibodies; anti-IL1R1 antibodies were used with Alexa 546 secondary antibody.

Western blots. Cells were lysed by M-PER Mammalian Protein Extraction Reagent (Pierce, IL), following the manufacturer's instructions. Protein concentration was determined with a Bradford assay (Bio-Rad). Thirty micrograms of total protein was loaded onto 6% or 10% SDS-PAGE gels according to the sizes of the proteins. After running, proteins was transferred to polyvinylidene difluoride membranes by a wet-transfer apparatus (Idea Scientific), and the blot was blocked in 10% nonfat dry milk in PBS for at least 1 h. Blots were probed with rabbit anti-IL1R1 at 1:200 dilution, rabbit anti-TLR5 at 1:500 dilution, mouse anti-TNFR1 at 1:500 dilution, rabbit anti MyD88 at 1:200 dilution, and mouse anti-ZO-1 at 1:1,000 dilution. Blots were then probed with horseradish peroxidase-tagged goat anti-rabbit IgG or horseradish peroxidase-tagged goat anti-mouse IgG accordingly. Bands were detected with the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

Statistics. Unpaired or paired *t*-tests were used to compare groups and effects, depending on the experiment (StatView, Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant. Data are presented as averages ± SD; *n* refers to the number of experiments.

RESULTS

HTE morphology and inflammatory receptors. Previous work (22, 28–30) showed that primary cells isolated from human tracheas and then grown on collagen-coated filters at an ALI in a medium containing Ultroser G typically exhibit a pseudostratified phenotype with two general cell types. Columnar cells (ciliated and goblet) have tight junctions attaching adjacent cells and apical cell membranes that contact both the airway surface liquid (ASL) and the basement membrane (30). Basal cells lie between the columnar cells and have membranes that attach to the basement membrane but do not reach the apical surface (30). Epithelial layers with $R_T > 100\ \Omega \cdot cm^2$ maintained an ALI, whereas those with $R_T < 100\ \Omega \cdot cm^2$ were usually incapable of maintaining an ALI. These results indicated that $R_T > 100\ \Omega \cdot cm^2$ was a critical level for these layers to exhibit intact tight junctions and polarization. HTE cultures routinely used for the present studies exhibited $R_T = 400–500\ \Omega \cdot cm^2$ and maintained a dry apical surface at the ALI.

We attempted to identify receptors and a receptor-related molecule that might be involved in responses to bacteria and the inflammatory cytokines with immunofluorescence. We used anti-centrin antibodies to identify ciliated cells, anti-MyD88 antibodies to identify cells expressing this key coupling protein in the TLR/IL1R pathway, and anti-IL1R1 antibody and anti-TNFR1 antibodies to localize these receptors. As shown in Figs. 1 and 2, HTE layers used here exhibited the pseudostratified phenotype with both columnar cells and basal cells. Centrin staining, characteristic of ciliated cells, was observed in 10–50% of the columnar cells (Fig. 1*A*) but in none of the basal cells (Fig. 1*B*). Although the percentage of ciliated cells in these layers is less than that observed in vivo (~90% ciliated), our preparations are similar to those used by other groups (4, 11). Ciliated, centrin-expressing columnar cells, nonciliated (goblet) columnar cells (Fig. 1*A*), and basal cells (Fig. 1*B*) all exhibited MyD88 staining. There was strong MyD88 staining in all the columnar cells of the monolayer (Fig. 1*A*), emphasized in *xz* sections (Fig. 1*C*), but there was also clear MyD88 staining in the basal cells (Fig. 1*B*).

Similar experiments were performed with anti-TLR5, anti-TNFR1, and anti-IL1R1 antibodies. Anti-ZO-1 antibodies

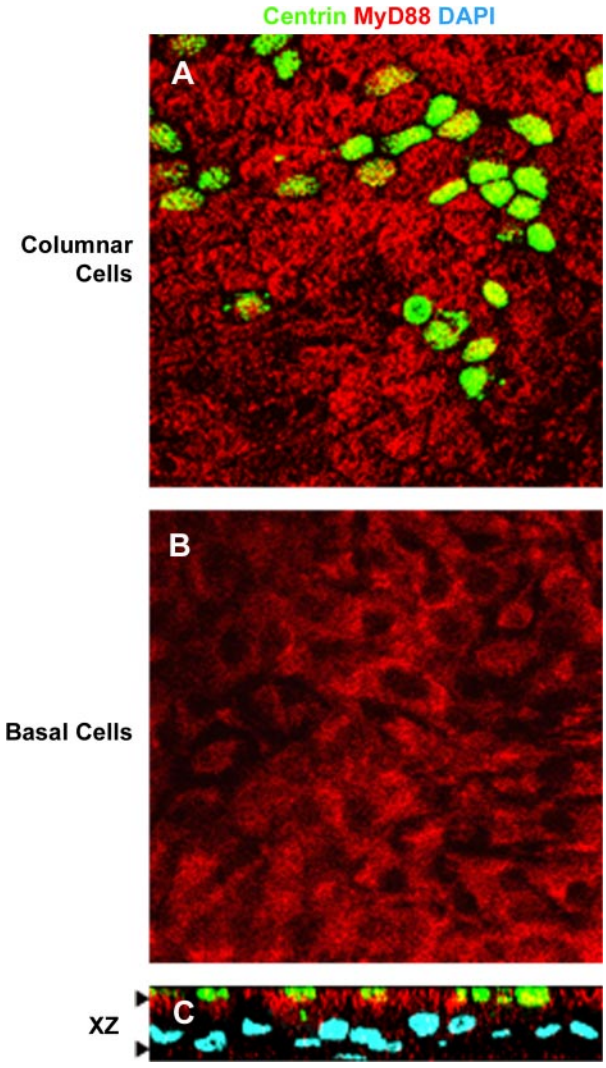

Fig. 1. Immunofluorescence localization of centrin and MyD88 in human tracheal epithelial (HTE) monolayers. HTE grown on filters were fixed, permeabilized, and stained for centrin (green), MyD88 (red), and nuclei [4′,6′-diamidino-2-phenylindole (DAPI), blue] and then examined in a confocal microscope. *xy* Sections were obtained for columnar (*A*) and basal (*B*) cell layers, marked with small arrowheads in *xz* section (*C*). DAPI (blue) staining shown in *xz* sections (*C*) localized nuclei of columnar and basal cells. Results are typical of 4 experiments.

were used in this set of experiments to identify tight junctions and the apical most regions of the cells in the same sections. TLR5, IL1R1, and TNFR1 were all identified in the apical most regions of the columnar cells, where ZO-1 was also observed (Fig. 2, *A–C*). These receptors were also observed in the middle, nuclear regions of the columnar cells (Fig. 2, *D–F*) as well as in the basal cells (Fig. 2, *G–I*). There was clear cytosolic staining for the receptors and MyD88, and some sections revealed staining that appeared to be localized to the membranes, but the close apposition of cells within the layers prevented positive membrane localization. Similar staining patterns were observed with HTE cells that had been stimulated with either flagellin or IL-1β + TNF-α on the apical and basolateral surfaces for 1 h before fixation (not shown). Western blots using the same antibodies used for the immunofluorescence studies demonstrated the specificity of the antibodies (Fig. 3).

Role of flagellin in P. aeruginosa activation of HTE layers during apical treatments. We tested for apical responsiveness of HTE layers to *P. aeruginosa* and flagellin. Typical results from a time course experiment in which the secretion of IL-8 into the basolateral solution was measured during control or during treatments with *P. aeruginosa* strain PAK, PAKΔfliC, flagellin, or flagellin + PAKΔfliC are shown in Fig. 4*A*. There was a small, slow increase in IL-8 secretion into the basolateral solution even in the absence of any treatment. Addition of PAK (40 MOI) to the apical surface caused larger amounts of IL-8 to be secreted into the basolateral solution. This dose of bacteria was chosen because work from several laboratories indicated this was likely to be a maximally effective dose (see, e.g., Ref. 13). Similar experiments were performed with purified flagellin at 1 μg/ml, a dose that yields maximal NF-κB activity of the recombinantly expressed mouse TLR5 in HEK cells (25). Both PAK- and flagellin-stimulated rates of IL-8 secretion were about five times larger than those measured from various cultured cell lines (see, e.g., Ref. 13), indicating that these HTE preparations were responding to bacteria or flagellin in the apical solution with robust IL-8 secretion.

The flagellin used for the present studies has been determined to be >95% pure, but we were concerned that a small contamination could still present a problem if HTE cells were particularly sensitive to LPS. As shown in Fig. 4, 10^{-6} g/ml flagellin elicited robust activation of IL-8 secretion. If this flagellin were contaminated solely with LPS to a level of 5%, there would be $\sim 5 \times 10^{-8}$ g/ml in our samples. We therefore compared the effects of 10^{-6} g/ml flagellin vs. 5×10^{-8} g/ml LPS on activation of NF-κB and IL-8 secretion by HTE cells. Cells were incubated with NF-κB adenovirus as described in MATERIALS AND METHODS, and the cells were then exposed, in separate experiments, on the apical or basolateral surface with flagellin, LPS, or flagellin + LPS for 4 h, when samples were taken from the basolateral solution for IL-8 measurement, and then the cells were lysed for luciferase measurements. As summarized in Table 1, flagellin from either the apical or basolateral surface elicited robust increases in NF-κB activation and IL-8 secretion. In contrast, there were only small (<25%) effects of LPS on IL-8 secretion and activation of NF-κB. In addition, there was no significant effect of LPS to augment the flagellin responses (i.e., flagellin vs. flagellin + LPS). We concluded that LPS contamination of our flagellin preparations was not contributing to the responses triggered in the HTE cells. Further experiments described below showed that, when comparing paired monolayers, effects of flagellin were larger when added to the basolateral compared with the apical side.

In contrast to the large responses stimulated by apical PAK or flagellin, addition of PAKΔfliC (40 MOI) to the apical surface did not stimulate IL-8 secretion from HTE cells into the basolateral solution above that observed in the control (untreated) monolayer. The combination of flagellin (1 μg/ml) + PAKΔfliC (40 MOI) elicited rates of IL-8 secretion similar, though somewhat smaller, to those elicited by flagellin alone. Averages from eight similar experiments in which paired comparisons were made among all the different treatments are summarized in Fig. 4*B*. These data showed that although there was variability in the magnitudes of IL-8 secreted into the basolateral solution during some of the treatments, HTE mono-

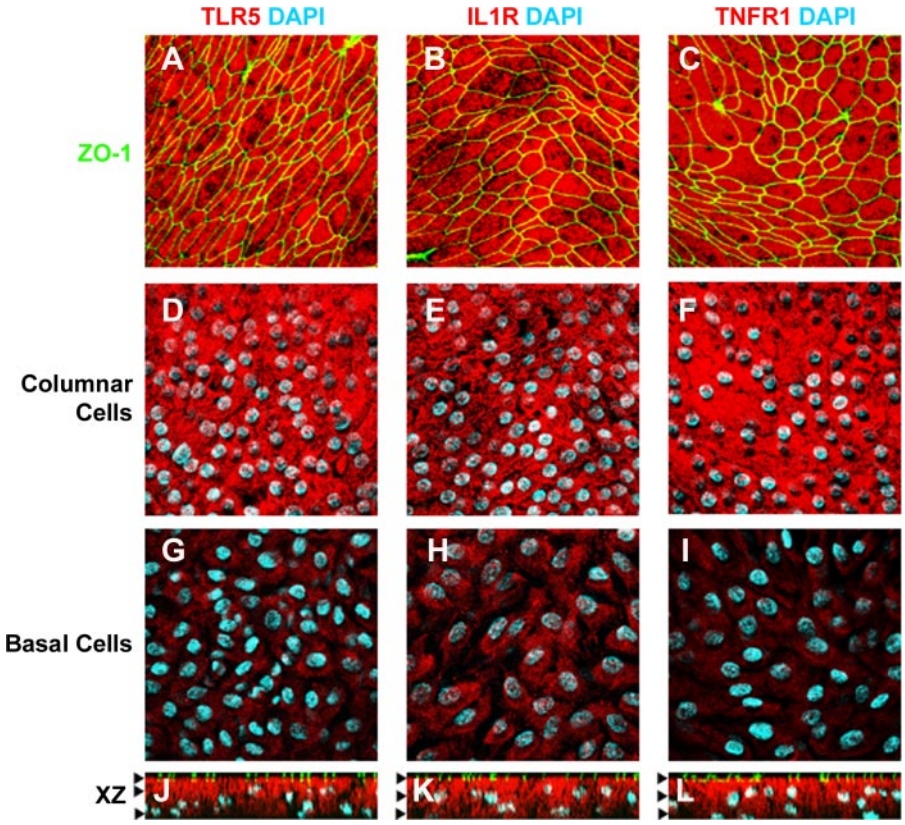

Fig. 2. Immunofluorescence localization of ZO-1, toll-like receptor (TLR)5, TNF-α receptor (TNFR)1, and IL-1 receptor (IL1R)1 in HTE monolayers. HTE grown on filters were fixed, permeabilized, stained and then examined in a confocal microscope. *xy* Sections were obtained for apical regions that exhibited ZO-1 (green) staining (*A–C*) and also for columnar (*D–F*) and basal (*G–I*) cell layers—these are marked with small arrowheads in *xz* sections (*J–L*). Staining was observed for TLR5 (red; *A*, *D*, *G*, *J*), TNFR1 (red; *B*, *E*, *H*, *K*), and IL1R1 (*C*, *F*, *I*, *L*) in apical and nuclear regions of columnar cells and in basal cells. Results are typical of 4 experiments.

layers increased their IL-8 secretion into the basolateral solution during treatment with apical PAK or flagellin, but there was no response to apical PAKΔfliC. These data also showed that flagellin expression by PAK was required to stimulate HTE responses during apical exposure; flagellin alone was sufficient to activate IL-8 secretion; and IL-8 secretion in response to PAKΔfliC + flagellin was somewhat smaller than that in response to flagellin alone.

We also tested effects of apical IL-1β (50 ng/ml) + TNF-α (100 ng/ml), which also induced a robust secretion of IL-8 into the basolateral solution of HTE layers similar in time course and magnitude to that elicited by apical PAK or flagellin (Fig. 4). These results are discussed in more detail below.

Similar experiments were performed to measure IL-8 secretion into the apical solution. Rates of IL-8 secretion into the apical solution in response to PAK, flagellin, or IL-1β + TNF-α were variable and only 2–20% of those into the basolateral solution (not shown). These data indicated that IL-8 secretion in response to apical inflammatory activators occurred more prominently into the basolateral than into the apical solution. We measured only basolateral secretion in subsequent experiments.

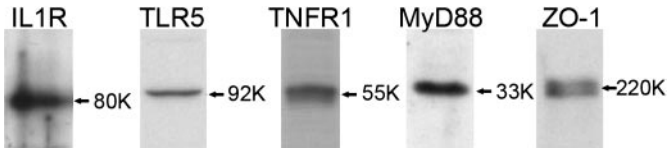

Fig. 3. Western blots for MyD88, TLR5, TNFR1, and IL1R1. Western immunoblot showing TLR5, MyD88, TNFR1, IL1R1, and ZO-1 in whole cell lysates of primary airway epithelial cell layer. Molecular masses in kDa are shown in each case. One representative experiment of 3 independent studies is shown.

Innate immune responses of HTE layers to apical vs. basolateral flagellin. The next experiments tested the relative responsiveness of HTE to flagellin on the apical vs. basolateral surface by measuring NF-κB activation, IL-8 secretion, and IL-8 gene expression. We first tested the concentration dependence of flagellin's effects on NF-κB activation and IL-8 secretion during apical vs. basolateral exposure. HTE layers were infected with adv-NF-κB-luc for 48 h and then treated with different concentrations of flagellin on either the apical or basolateral surface. Samples were collected from the basolateral solution for measurement of IL-8 secretion, followed by cell lysis and processing for luciferase activity. Both apical and basolateral flagellin induced dose-dependent increases in NF-κB activation, with a threshold at $\sim 10^{-8}$ g/ml and sequentially larger effects at 10^{-7} and 10^{-6} g/ml (Fig. 5). Similar dose dependence was observed for basolateral and apical flagellin on IL-8 secretion into the basolateral solution, although, again, basolateral flagellin induced larger IL-8 secretion compared with apical flagellin; results from typical experiments are shown in Fig. 6. Average results for apical vs. basolateral effects of the maximal dose of flagellin (10^{-6} g/ml) on IL-8 secretion and IL-8 gene expression are shown in Fig. 7. These results showed that the host responses of HTE cells were larger when flagellin was present on the basolateral compared with the apical side, and the dose dependence of activation by flagellin was similar to that exhibited by recombinant TLR5 expressed in HEK cells (25).

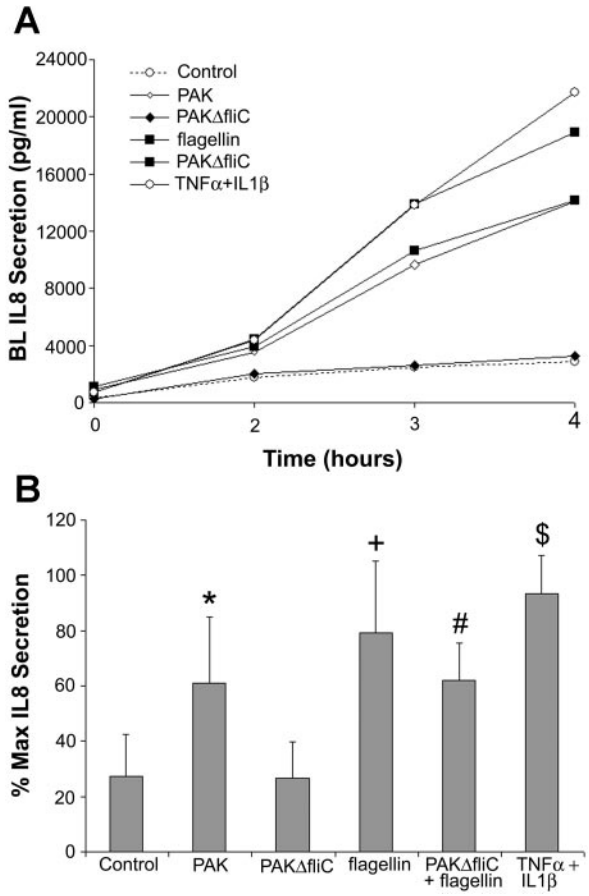

Fig. 4. IL-8 secretion into basolateral solution in response to apical PAK, PAKΔfliC, flagellin, and PAKΔfliC + flagellin. HTE monolayers grown on permeable supports at an air-liquid interface were exposed to PAK [40 multiplicity of infection (MOI)], PAKΔfliC (40 MOI), flagellin (10^{-6} g/ml), or flagellin + PAKΔfliC on the apical surface, and samples were taken from the basolateral (BL) solution for measurements of IL-8 secretion with ELISA. *A*: time course of IL-8 secretion for 1 typical experiment (of 5 similar). *B*: normalized rates of IL-8 secretion during 4-h treatment with PAK, PAKΔfliC, flagellin, and flagellin + PAKΔfliC on the apical surface. IL-8 secretions over 4 h were normalized by dividing by the maximal amount observed during each individual experiment. Apical treatment of HTE with PAK or flagellin stimulated IL-8 secretion into the basolateral solution, whereas PAKΔfliC had no stimulatory effect. Data are averages ± SD for 8 experiments. Statistically significant differences: *PAK vs. control ($P < 0.0.01$); +flagellin vs. control ($P < 0.001$); #flagellin + PAKΔfliC vs. control ($P < 0.01$); $IL-1β + TNF-α vs. control ($P < 0.001$). PAKΔfliC vs. control, $P > 0.05$.

Innate immune responses of HTE cells to apical vs. basolateral IL-1β + TNF-α. The next experiments tested the relative responsiveness of HTE layers to IL-1β (50 ng/ml) + TNF-α (100 ng/ml) on the apical vs. basolateral surface by measuring NF-κB activation, IL-8 secretion, and IL-8 gene expression. As summarized in Fig. 8, there was greater activation of NF-κB during basolateral compared with apical exposure to IL-1β + TNF-α. In a second set of experiments, samples were taken from the basolateral solution of HTE cells at *time* = 0 and then after 4 h for measurement of IL-8 secretion by ELISA, a time frame that was sufficient to elicit maximal IL-8 secretion (see Fig. 4*A*). At the end of the experiments cells were processed for IL-8 gene expression with QPCR. Basolateral IL-1β + TNF-α elicited larger effects on both IL-8 secretion (Fig. 9*A*) and gene expression (Fig. 9*B*) compared with apical addition of IL-1β + TNF-α.

Table 1. *Effects of LPS and flagellin on NF-κB activation and IL-8 secretion by HTE monolayers*

	NF-κB Ratio to control	IL-8, pg/ml
Control		
Expt. 1 AP	1.0	8,421±482
Expt. 2 AP	1.0	10,275±967
Expt. 3 BL	1.0	7,628±1,087
Expt. 4 BL	1.0	8,173±1,239
Flagellin		
Expt. 1 AP	6.2±0.1	41,664±3,369
Expt. 2 AP	3.6±0.1	30,598±1,760
Expt. 3 BL	12.3±0.1	30,301±4,875
Expt. 4 BL	6.1±0.0	34,018±860
LPS		
Expt. 1 AP	1.2±0.0	10,201±1,109
Expt. 2 AP	1.2±0.0	11,954±2,428
Expt. 3 BL	1.4±0.0	8,739±885
Expt. 4 BL	0.9±0.0	11,891±989
LPS + flagellin		
Expt. 1 AP	6.5±0.2	53,528±4,558
Expt. 2 AP	NA	NA
Expt. 3 BL	7.8±0.1	24,528±5,145
Expt. 4 BL	4.9±0.0	27,165±1,150

Values are averages ± SD for 3 replicates of 4 experiments, 2 during apical (AP) additions and 3 during basolateral (BL) additions. Human tracheal epithelial (HTE) monolayers were exposed to NF-κB luciferase adenovirus for 48 h and then left untreated or treated on the AP (*experiments 1* and *2*) or BL surface (*experiments 3* and *4*) with LPS (5×10^{-8} g/ml), flagellin (10^{-6} g/ml), or flagellin + LPS for 4 h. Luciferase was measured in relative light units and expressed relative to the control (untreated) cells. IL-8 was assayed from samples taken from the basolateral solution bathing the HTE monolayers. NA, treatment not performed.

Identification of flagellin- and IL-1β + TNF-α-responsive cells. One possible explanation for the smaller responses to apical vs. basolateral flagellin and IL-1β + TNF-α was that the appropriate flagellin and inflammatory cytokine receptors were present on the apical membranes of only some of the cells but on the basolateral membranes of all the cells. We tested this possibility by monitoring NF-κB migration into the nucleus of

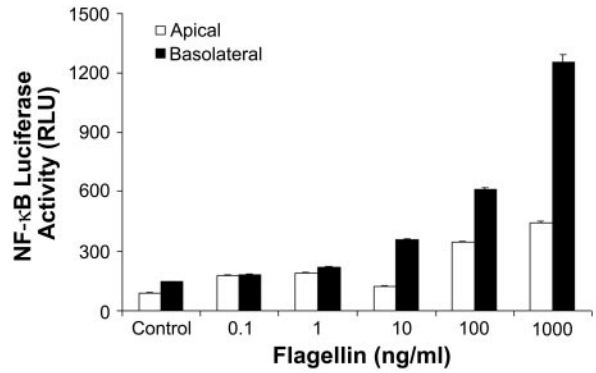

Fig. 5. Effects of apical vs. basolateral flagellin concentration ([flagellin]) on NF-κB activation. HTE monolayers grown at air-liquid interface (ALI) were treated with adv-NF-κB-luc on the basolateral surface for 48 h and then incubated with different [flagellin] on the apical or basolateral surface for 4 h before luciferase assays were performed on the cell extracts. Flagellin caused dose-dependent increases in NF-κB activity [expressed in relative light units (RLU) from luciferase assay], with thresholds occurring at ~10^{-8} g/ml for both apical and basolateral exposure. There was a larger effect of flagellin on NF-κB activity during basolateral compared with apical application. Data are averages ± SD for 3 paired experiments.

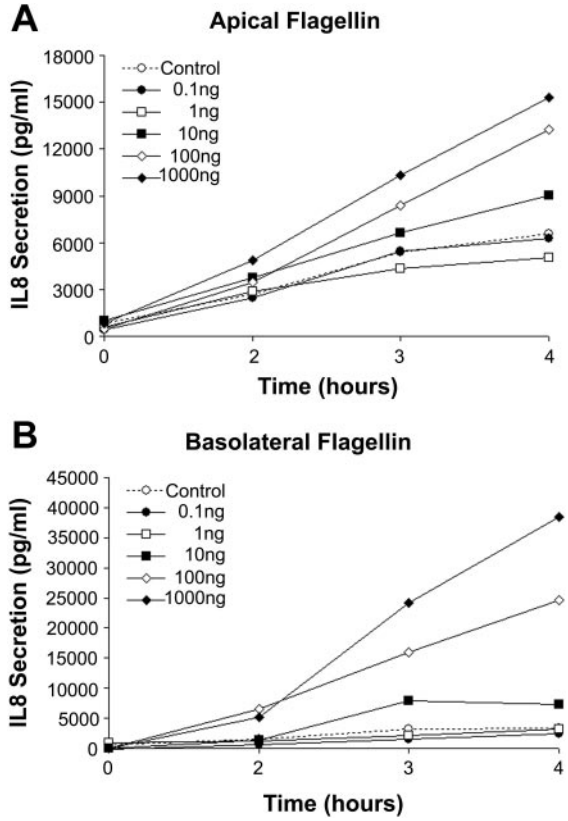

Fig. 6. Effects of apical vs. basolateral [flagellin] on IL-8 secretion into the basolateral solution. IL-8 secretion into the basolateral solution was measured by ELISA, and time courses are shown for apical (*A*) and basolateral (*B*) application of flagellin. Basolateral application of flagellin elicited larger IL-8 secretion during basolateral vs. apical exposure. Results are typical of 3 similar experiments.

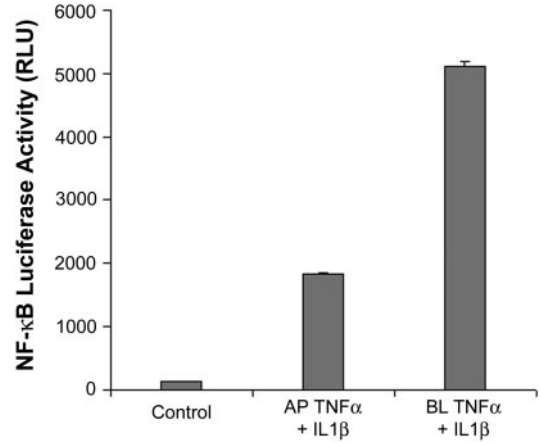

Fig. 8. Effects of apical vs. basolateral IL-1β + TNF-α on NF-κB activation in HTE cells. Confluent monolayers grown on permeable supports at ALI were treated with adv-NF-κB-luc on the basolateral surface for 48 h and then incubated on the apical or basolateral surface with IL-1β (50 ng/ml) + TNF-α (100 ng/ml) for 4 h before luciferase assays were performed on the cell extracts (expressed in RLU). There was a larger effect of basolateral compared with apical IL-1β + TNF-α on NF-κB activation. Data are averages ± SD for 3 paired experiments. Statistical comparisons: $P < 0.05$ for apical IL-1β + TNF-α vs. control, basolateral IL-1β + TNF-α vs. control, and apical vs. basolateral IL-1β + TNF-α.

columnar and basal cells. HTE cells were exposed to flagellin or IL-1β + TNF-α on the apical or basolateral surface for 1 h, and the cells were then fixed, permeabilized, stained with both anti-NF-κB antibodies and DAPI (to identify nuclei), and examined for nuclear migration of NF-κB as performed previously (1). Typical images of columnar cells and basal cells from HTE monolayers that had been left untreated (control) or exposed to apical flagellin or basal flagellin are shown in Fig. 10. These layers expressed typical numbers of centrin-expressing, ciliated cells like those shown in Fig. 1 (not shown). In control, untreated monolayers, NF-κB was present in the cytosol but not in the nuclei of all columnar (Fig. 10, *A–C*) and basal (Fig. 10, *D–F*) cells. Treatment with flagellin (1 μg/ml) in the apical solution caused NF-κB to migrate into the nucleus of almost all columnar cells (Fig. 10, *J–L*) but very few basal cells (Fig. 10, *M–O*). Treatment with flagellin on the basolateral surface caused consistent activation of NF-κB in all columnar cells (Fig. 10*U*) and basal cells (Fig. 10, *V–X*).

Similar results were obtained with apical vs. basolateral TNF-α + IL-1β, i.e., apical TNF-α + IL-1β caused NF-κB to

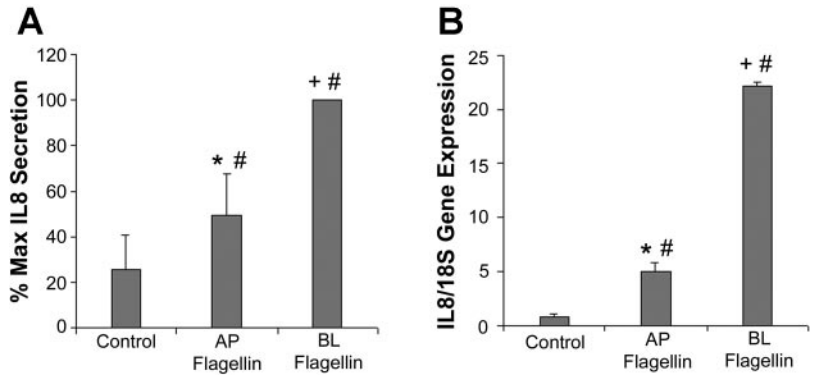

Fig. 7. Apical vs. basolateral flagellin (10^{-6} g/ml) on IL-8 secretion and IL-8 gene expression. Flagellin (1 μg/ml) was added to either the apical (AP) or basolateral (BL) surface of HTE cells for 4 h. Samples were taken from the basolateral solution for measurement of IL-8 secretion (ELISA). At the end of the experiments cells were isolated, and RNA was extracted for measurement of IL-8 and ribosomal 18S gene expression [quantitative PCR (QPCR)]. *A*: HTE monolayers secreted an average of 2,497 ± 1,335 pg IL-8 after 4 h under control conditions (no treatment), and this increased by a factor of ~3 during apical application of flagellin and by a factor of ~6 during basolateral flagellin treatment. Data are averages ± SD for 5 paired experiments. *B*: IL-8-to-18S gene expression ratio was normalized to the control condition. There was a larger increase in IL-8-to-18S ratio during basaolateral compared with apical flagellin treatment. Data are averages ± SD for 3 paired experiments. Statistically significant differences: *apical flagellin vs. control ($P < 0.05$); $^{+}$basolateral flagellin vs. control ($P < 0.01$); $^{\#}$apical flagellin vs. basolateral flagellin ($P < 0.01$).

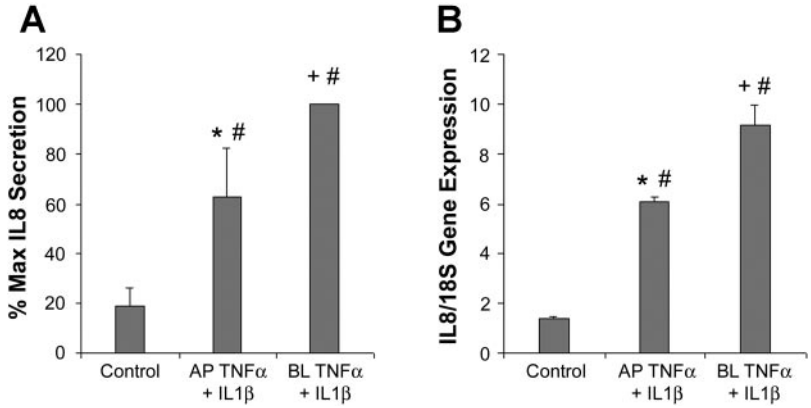

Fig. 9. Effects of apical vs. basolateral IL-1β + TNF-α on IL-8 secretion and IL-8 gene expression by HTE monolayers. Confluent monolayers grown on permeable supports at ALI were exposed to IL-1β (50 ng/ml) + TNF (100 ng/ml) on either the apical or basolateral surface for 4 h. Samples were taken from the basolateral solution at $t = 0$ and after 4 h for measurement of IL-8 secretion by ELISA (*A*) and then processed for IL-8 gene expression with QPCR (*B*). *A*: HTE monolayers secreted an average of 2,589 ± 2,010 pg IL-8 after 4 h under control conditions (no treatment), and this increased by a factor of ~3 during apical application of IL-1β + TNF-α and by a factor of ~5 during basolateral IL-1β + TNF-α treatment. *B*: IL8/18S gene expression ratio was normalized to the control condition. There was a larger increase in IL8/18S ratio during basolateral compared with apical TNF-α + IL-1β treatment. Data for *A* and *B* are averages ± SD for 6 experiments. Data are averages ± SD for 6 experiments. Statistically significant differences: *apical IL-1β + TNF-α vs. control ($P < 0.001$); +basolateral IL-1β + TNF-α vs. control ($P < 0.001$); #apical IL-1β + TNF-α vs. basolateral IL-1β + TNF-α ($P < 0.001$).

migrate into the nucleus of most columnar cells (Fig. 11, *A–C*) but few basal cells (Fig. 11, *D–F*). Treatment with TNF-α + IL-1β on the basolateral surface caused consistent activation of NF-κB in both columnar cells (Fig. 11, *J–L*) and basal cells (Fig. 11, *M–O*).

Results from these NF-κB localization experiments were quantitated by counting nuclei (DAPI) in which there was clear NF-κB accumulation in the nucleus. Data for nuclear migration of columnar and basal cells during apical vs. basolateral treatment with either flagellin or TNF-α + IL-1β have been summarized in Table 2 and show that exposure to maximally stimulatory doses of either flagellin or TNF-α + IL-1β led to NF-κB activation in all columnar cells but no basal cells. Exposure to the stimulants on the basolateral surface activated (nuclear migration of NF-κB) nearly all columnar and basal cells.

DISCUSSION

Flagellin is a necessary and sufficient P. aeruginosa activator of innate immune response in columnar HTE cells. One major conclusion from these studies was that flagellin is both necessary and sufficient for apical *P. aeruginosa* to activate an innate immune response of primary, polarized human airway epithelial cells. Thus wild-type PAK and flagellin activated equal rates of IL-8 secretion, PAKΔfliC had no effect, and flagellin + PAKΔfliC elicited IL-8 secretion roughly equal to that induced by PAK. Furthermore, flagellin alone elicited all aspects associated with a typical innate immune response: NF-κB activation and migration to the nucleus, followed by increased IL-8 gene expression and secretion of IL-8 into the basolateral solution. The cells also secreted IL-8 into the apical solution, but this secretion was much less consistent than that into the basolateral solution.

Although flagellin preparations can sometimes be contaminated with LPS, we have several reasons for thinking this was not a problem in the present experiments. First, data in Fig. 4 show that PAKΔfliC, which have LPS, do not activate airway epithelial cells to secrete IL-8 above that of the controls, whereas flagellin + PAKΔfliC activate cells to produce IL-8 at rates that are roughly equal to epithelia treated with flagellin alone. This result indicated that intact PAKΔfliC with a normal complement of LPS but without flagellin did not stimulate IL-8, whereas adding purified flagellin to PAKΔfliC stimulated IL-8 secretion. Second, flagellin in our preparations has been determined to be >95% pure, and exogenous LPS addition in amounts that might be expected if the entire contamination of our flagellin preparations were due to LPS (i.e., 5×10^{-8} g/ml, $\sim 2 \times 10^{-8}$ M) elicited only very small (<25% increase) effects on NF-κB activation and IL-8 secretion. In contrast, flagellin (10^{-6} g/ml or 2.5×10^{-8} M) caused four- to sixfold increases in NF-κB activation and IL-8 secretion. Furthermore, activation of NF-κB and IL-8 secretion was the same for flagellin and flagellin + LPS. Our results were therefore consistent with experiments of Jia et al. (11), who found that primary airway epithelial cells were insensitive to LPS and only responded sensitively (pM concentrations) to LPS after the cells had been transfected with the TLR4 coreceptor MD2. Gioannini et al. (8) also showed that endotoxin activation of TLR4 occurred in the picomolar range when the MD2-endotoxin complex interacted with TLR4-CD14 (8). An implication of these studies is that primary airway epithelia are insensitive to LPS because TLR4 is not operative due to absence of critical MD2 expression. Thus it seems likely that *P. aeruginosa* flagellin is the most important component of *P. aeruginosa* activating airway epithelia from the apical surface.

Our confocal images showed MyD88 prominently localized to the apical regions of all the columnar cells, both ciliated and nonciliated, but there was insufficient resolution to determine whether this TLR-associated protein was actually expressed in the apical membranes. However, the relatively rapid responses to apical flagellin (NF-κB migrated to the nucleus of these cells within 1 h) showed that at least some of the MyD88 and any associated receptors were likely to be in the apical membranes of all the columnar cells. These results indicated that the

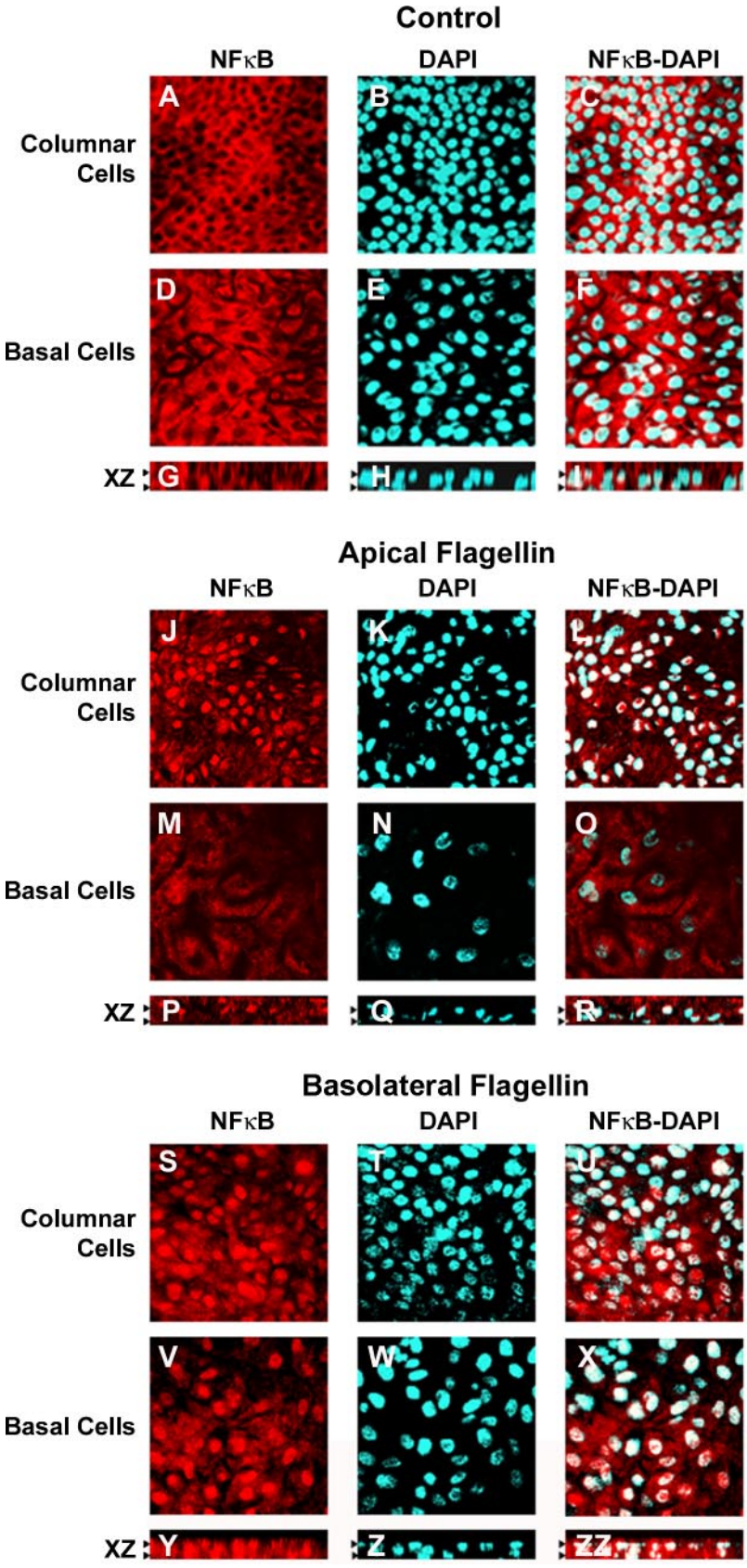

Fig. 10. Effects of apical vs. basolateral flagellin on NF-κB activation in columnar and basal cells in HTE monolayers. HTE were left untreated (control) or exposed to flagellin (1 μg/ml) on the apical or basolateral surface for 1 h. Cells were then fixed, permeabilized, stained with DAPI (blue), anti-NF-κB, and secondary (red) antibodies and examined in the confocal microscope. *xy* Sections were taken through the nuclear region of the columnar cells (*A–C*; *J–L*; *S–U*) and the basal cells (*D–F*; *M–O*; *I*, *W*, *X*). Arrowheads in *xz* reconstructions (*G–I*; *P–R*; *Y–ZZ*) show regions where *xy* slices were taken. Inactive cells had DAPI-stained nuclei, but NF-κB remained in the cytosol, excluded from the nucleus. Activated cells exhibited NF-κB staining in the nucleus. There were hardly any activated surface or basal cells in the control condition. Apical flagellin activated most columnar cells but no basal cells. Basolateral flagellin activated both basal cells and columnar cells. Results are typical of at least 4 experiments for each condition.

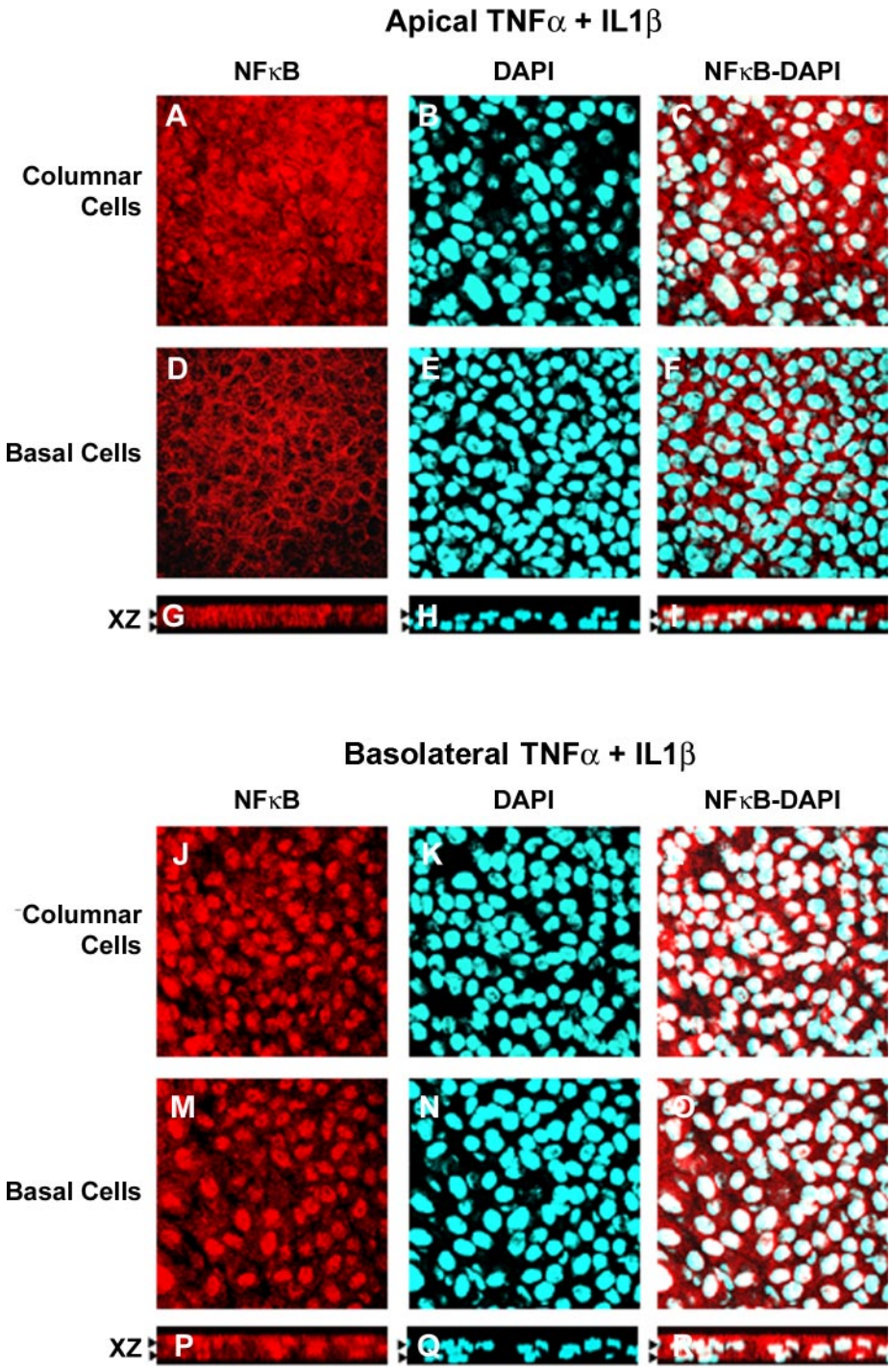

Fig. 11. Effects of apical vs. basolateral TNF-α + IL-1β on NF-κB activation in columnar and basal cells in HTE monolayers. HTE were exposed to TNF-α (50 ng/ml) + IL-1β (100 ng/ml) on the apical or basolateral surface for 1 h. Cells were then fixed, permeabilized, stained with DAPI (blue), anti-NF-κB, and secondary (red) antibodies, and examined in the confocal microscope. *xy* Sections were taken through the nuclear region of the columnar cells (*A–C*, *J–L*) and the basal cells (*D–F*, *M–O*). Arrowheads in *xz* reconstructions (*G–I*, *P–R*) show regions where *xy* slices were taken. Inactive cells had DAPI-stained nuclei, but NF-κB remained in the cytosol, excluded from the nucleus. Activated cells exhibited NF-κB staining in the nucleus. Apical TNF-α + IL-1β activated most columnar cells but no basal cells. Basolateral TNF-α + IL-1β activated both columnar cells and basal cells. Results are typical of 5 experiments for each condition.

columnar epithelial cells were poised to respond to monomeric flagellin released or shed from *P. aeruginosa* or other bacteria into the ASL.

Assuming that each square centimeter of airway epithelium contains ~10^6 epithelial cells and is covered by 1 μl of ASL (depth = 10 μm) (12), that monomeric flagellin from intact bacteria has a molecular mass of ~50 kDa (22), and that there are 20,000 flagellin monomers per flagellum (Ramphal R, personal communication), we calculate that if each *P. aeruginosa* released or shed all of its flagellin monomers from one flagellum into the ASL, then the airway epithelium would have a threshold sensitivity to the presence of ~500 bacteria per milliliter of ASL, i.e., on average <1 bacterium per microliter of ASL in contact with 10^6 epithelial cells. CF patients can have up to 10^8 *P. aeruginosa* per milliliter of sputum (i.e., 10^5 bacteria/μl ASL covering 10^6 epithelial cells). We conclude that a host response by the airway epithelial cells will be a natural sequela if only a few bacteria accumulate in and shed all their flagellin into the ASL. This calculation also emphasizes the importance of mucociliary escalator and antimicrobial factors in keeping the ASL clean and preventing inflammatory reactions from being triggered.

Table 2. *Effects of apical vs. basolateral flagellin and TNF-α + IL-1β on nuclear migration (activation) of NF-κB of columnar and basal cells of HTE monolayers*

Treatment	*n*	Cells with Nuclear NF-κB	Total Cells	%Activated
Control				
Columnar cells	8	1	748	0.1
Basal cells	8	0	429	0
AP flagellin				
Columnar cells	11	1,238	1,324	94
Basal cells	11	1	684	0.2
BL flagellin				
Columnar cells	6	427	454	94
Basal cells	4	148	148	100
AP IL-1β + TNF-α				
Columnar cells	5	496	520	95
Basal cells	5	1	489	0
BL IL-1β + TNF-α				
Columnar cells	5	828	871	95
Basal cells	5	654	659	99

n, Number of different sections examined from 2–6 different HTE monolayers. HTE were left untreated (control) or exposed to flagellin (1 μg/ml) on the apical or basolateral surface for 1 h or exposed to IL-1β (50 ng/ml) + TNF-α (100 ng/ml) on the apical or basolateral side. Cells were then fixed, permeabilized, stained with 4′, 6′-diamidino-2-phenylindole (DAPI) and anti-NF-κB antibody, and finally examined in the confocal microscope (as in Fig. 7). Total number of columnar and basal cells was determined from number of nuclei (identified by DAPI staining) in each region examined, and number of columnar and basal activated cells was determined as those with NF-κB in the nucleus. The apparent differences in numbers of columnar and basal cells resulted from the fact that NF-κB staining in the basal cells was often much less intense than that in the columnar cells, so that it was impossible to determine nuclear vs. cytosolic localization of NF-κB. We counted only cells in which NF-κB staining was distinct enough to permit nuclear or cytosolic localization, and there were fewer of these than in columnar cells.

Another important conclusion from these experiments is that the columnar cells in the airway epithelium alone have the capacity to respond with a vigorous innate immune response to the presence of bacteria in the ASL, even in the absence of other cell types that will be present in vivo, e.g., glandular cells, macrophages, and dendritic cells. These results therefore disprove our previous proposal (10) that macrophages or some other cell type not present in our CF15 cell culture model may have been important for triggering the inflammatory response to *P. aeruginosa* in the intact airways. One possible explanation for our previous findings of apparent insensitivity of CF15 cells to apical *P. aeruginosa* may be that apical flagellin receptors are missing in these cells but present in primary cells. This type of explanation could also explain the apparent contradiction regarding TLR5 on the apical vs. basolateral surface of intestinal cells: T84 cells responded only to basolateral flagellin and exhibited basolateral TLR5 (7), whereas primary intestinal epithelial cells exhibited apical flagellin-dependent responses and apical TLR5 (2).

TNF-α + IL-1β also activate innate immune responses in columnar HTE cells. A second major finding to emerge from the present studies was that apical TNF-α + IL-1β elicited activation of innate immune responses similar to those elicited by apical flagellin: activation of NF-κB in both the ciliated and nonciliated columnar epithelial cells, followed by increased IL-8 gene expression and secretion of IL-8 into the basolateral solution. There was also prominent staining for IL1R1 and TNFR1 in all the columnar cells, although, similar to the MyD88 staining, it was impossible to localize this staining to the apical membrane. These data indicated that any macrophages patrolling the upper airways that respond to *P. aeruginosa* by secreting TNF-α + IL-1β will contribute to activation of inflammatory processes of the columnar airway epithelial cells, similar to those triggered by the bacteria. This is likely the circumstance that will exist in vivo.

Apical flagellin or TNF-α + IL-1β activates columnar cells but not basal cells; basolateral flagellin or TNF-α + IL-1β activates columnar cells and basal cells. Apical flagellin or TNF-α + IL-1β caused selective activation (cytosol-to-nucleus migration of NF-κB) of only columnar cells, whereas basolateral flagellin or TNF-α + IL-1β activated NF-κB in both columnar cells and basal cells. These results were consistent with the idea that columnar cells and their tight junctions prevented flagellin or cytokines in the ASL from contacting and activating the basal cells. These results also likely explain the fact that basolateral flagellin or IL-1β + TNF-α elicited 1.5- to 4-fold larger activations of NF-κB and IL-8 gene expression and secretion compared with apical applications: basolateral treatments activated NF-κB and innate immune responses in both columnar cells and basal cells, whereas apical treatments led to responses only in columnar cells. An obvious implication of these results is that airway epithelial tissue will exhibit an exaggerated innate immune response during conditions in which the bacteria or inflammatory cytokines gain access to the basolateral membranes, e.g., during epithelial trauma or influx of phagocytes to the infected region. The effects of epithelial trauma on the inflammatory responses to bacteria could be tested directly with either scrape wounding or chemical treatments of open tight junctions.

Are receptors for flagellin, TNF-α, and IL-1β present in both apical and basolateral membranes? Although the responses to basolateral flagellin by the columnar and basal cells were larger than during apical treatments, the responses were qualitatively similar: both apical and basolateral treatments increased NF-κB activation and IL-8 expression and secretion, and there were similar dose-dependent effects of apical vs. basolateral flagellin on NF-κB activation and IL-8 secretion, with thresholds of $\sim 10^{-8}$ g/ml and steadily increasing activation to 10^{-6} g/ml. The apparently higher sensitivity of the epithelial cells to basolaterally vs. apically applied flagellin (Fig. 5) was perhaps due to the fact that during basolateral applications flagellin was activating both basal and columnar cells, which likely responded with similar dose dependence in activating NF-κB. In contrast, during apical application the basal cells did not respond, and these cells may therefore have provided a "background" of nonresponding cells that reduced total NF-κB activity compared with the activity in the columnar cells, which were responding to the flagellin. The dose dependence for flagellin activation of HTE cells was similar to the dose dependence of flagellin on NF-κB activation in TLR5-expressing HEK cells (25). The responses to apical and basolateral TNF-α + IL-1β were also similar to each other.

One explanation for these results is that the same receptors for these agonists were present on apical and basolateral membranes of both columnar and basal cells. Consistent with these findings, all columnar cells expressed MyD88 and also TLR5, TNFR1, and IL1R1. Although staining for these components was strongest in the columnar cells, there was also significant staining in basal cells. Some of the differential

staining might be explained by differential antibody penetration into the surface vs. basal cells during preparation for microscopic examination. Further studies will be required to settle this issue.

Although the physiological responses of the cells to flagellin and IL-1β and TNF-α indicate that the appropriate receptors were present in the apical and basolateral membranes of the cells, the confocal images did not yield good enough resolution to determine membrane localization. It is possible that the receptors are expressed in relatively small numbers in the plasma membrane, at least compared with the numbers in internal compartments. Localizing membrane receptors to the plasma membrane is particularly difficult in confluent epithelia because adjacent cells are tightly packed, membranes are folded, and there is expression in both membranes and organelles. Previous experiments have shown that basolateral IL-1β triggers changes in columnar cell tight junction structure and function, consistent with the idea that IL1R1 is present and active in the basolateral membranes of columnar cells (4). Together, these data indicate that the same flagellin and cytokine receptors may operate in both apical and basolateral membranes of columnar cells and also in the membranes of nonpolarized basal cells.

Previous experiments have shown apical membrane localization of TLR2 and also, after long-term flagellin treatment, TLR5, which appeared to move from the cytosol to the apical membrane (1). The staining patterns for MyD88, TLR5, TNFR1, and IL1R1 in HTE cells were the same in both untreated and flagellin- or TNF-α + IL-1β-treated columnar and basal cells. These data indicated that the trafficking of these inflammation-associated receptors could be different in different cells. In addition, coreceptors like asialoGM1 (see Refs. 1, 17, 18) could modify the activities of the TLR2 or TLR5 receptors at the apical and/or basolateral membranes of the cells. This might explain the somewhat lower sensitivity of HTE cells to apical vs. basolateral flagellin. It will be important to characterize the trafficking of the flagellin and cytokine receptors and coreceptors to the apical and basolateral membranes of both columnar and basal cells in HTE. It will be similarly important to determine trafficking of these receptors in other epithelia that are exposed to apical bacteria and phagocytes, e.g., intestine.

Although we have shown that TLR5 is present in both columnar and basal cells and that the cells respond to flagellin from the apical and basolateral surfaces, the specific roles of TLR5 vs. TLR2 in these responses of HTE cells remain to be clarified (e.g., see Ref 1). However, we can exclude two possible explanations for our results. It has been proposed for the intestine that apical *Salmonella typhimurium* elicits inflammatory responses by flagellin gaining access to the basolateral surfaces of the cells where TLR5 is located (7). This is unlikely to occur in the airways because the basal cells of HTE did not become activated by apical flagellin or *P. aeruginosa*, although the basal cells were readily activated when flagellin was added to the solution bathing the basolateral surface of the epithelium. It is similarly unlikely that uptake of *P. aeruginosa* by airway epithelial cells is involved (12), because this occurs only infrequently (20) and therefore could not account for the nearly uniform responses by all columnar cells. Instead, our data indicate that the same or at least very similar receptors and signaling pathways must be operable at the apical and basolateral membranes of all columnar cells and also in the membranes of the nonpolarized basal cells. Columnar cells are activated selectively during the presence of bacteria or activated phagocytes in the ASL likely because tight junctions prevent access of flagellin and proinflammatory cytokines to the basal cells. It seems likely that both columnar cells and basal cells are activated during epithelial trauma when inflammatory stimulants gain access to the serosal surface of the airway epithelial tissue. This will be the subject of future work.

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