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TLR2 Regulates Gap Junction Intercellular Communication in Airway Cells

Francis J. Martin* and Alice S. Prince2*†

The innate immune response to inhaled bacteria, such as the opportunistic Pseudomonas aeruginosa, is initiated by TLR2 displayed on the apical surface of airway epithelial cells. Activation of TLR2 is accompanied by an immediate Ca2+ flux that is both necessary and sufficient to stimulate NF-κB and MAPK proinflammatory signaling to recruit and activate polymorphonuclear leukocytes in the airway. In human airway cells, gap junction channels were found to provide a regulated conduit for the movement of Ca2+ from cell to cell. In response to TLR2 stimulation, by either lipid agonists or P. aeruginosa, gap junctions functioned to transiently amplify proinflammatory signaling by communicating Ca2+ fluxes from stimulated to adjacent, non-stimulated cells thus increasing epithelial CXCL8 production. P. aeruginosa stimulation also induced tyrosine phosphorylation of connexin 43 and association with c-Src, events linked to the closure of these channels. By 4 h postbacterial stimulation, gap junction communication was decreased indicating an autoregulatory control of the connexins. Thus, gap junction channels comprised of connexin 43 and other connexins in airway cells provide a mechanism to coordinate and regulate the epithelial immune response even in the absence of signals from the immune system. The Journal of Immunology, 2008, 180: 4986–4993.

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Materials and Methods

Reagents

Pam-AM, Cys-Ser-Lys-P (P3C) was purchased from EMC Microcollections. Calcein-AM, Vybrant DiI, thapsigargin, and Fluoro-3/AM were purchased from Molecular Probes. Aphyrase, carbenoxolone, 18-glyceric acid, pluronnic acid, monoclonal anti-β-actin Ab, and polyclonal anti-Cx43 Ab (for immunoprecipitation) were purchased from Sigma-Aldrich. Mouse anti-phosphotyrosine clone 4G10 was purchased from Upstate Biotechnology. HRP-conjugated goat anti-β-actin Ab was obtained from Bethyl Laboratories and used to detect the FLAG moiety. A mouse anti-Cx43 Ab was purchased from BD Biosciences and used for Western blotting. The activated c-Src (Tyr416) Ab was obtained from Cell Signaling. Cx43 (H-150), caveolin-1 (N-20), TLR2 (H-175), and c-Src (B-12 and SC-18 (for immunoprecipitation)) Abs were obtained from Santa Cruz Biotechnology. Unless otherwise noted, additional reagents were obtained from Invitrogen Life Technologies. The pcDNA 3.1 and NF-κB luciferase reporter plasmids were gifts from J. D. Li (University of Rochester, Rochester, NY).

Cell culture and bacteria

1HAEo human airway epithelial cell lines (D. Gruenert, Pacific Medical Center Research Institute, San Francisco, CA) were grown as previously described (4, 23). Airway cells were transfected using FuGENE 6 (Roche) according to the manufacturer’s instructions. Forty-eight hours after transfection with pcDNA3.1 constructs, cells were grown under neomycin selection. Experiments using these cell lines were performed after cells had been grown under selection for at least 2 wk. 1HAEo cells expressing Flag-TLR2 have been previously described (19). Pseudomonas aeruginosa PAO1 was grown on Luria-Bertani agar plates, resuspended in 1HAEo complete medium or MEM and heat killed for 1 h at 60°C.

Mouse infections

C57BL/6 mice (7–10 days old) were intranasally inoculated with 106 CFU P. aeruginosa PAO1. The mice were administered 18g glyceric acid (5 mg/kg in DMSO/PBS) or vehicle by i.p. injection 12 h prior, 2 h prior, and immediately following bacterial inoculation. After 4 h of infection, mice were euthanized with pentobarbital. Lungs were collected and cell suspensions were subjected to red cell lysis, blocking of nonspecific binding by incubation with 10% normal mouse serum and mouse Fc block (clone 24G2; BD Biosciences), and staining with FITC-conjugated anti-Ly6G/Ly6C (clone RB6-8C5; BD Biosciences) and PE-conjugated anti-CD45 (clone 30-F11; Caltag Laboratories) or appropriate isotype controls. Cells were gated on their forward scatter/side scatter properties and analyzed for expression of both CD45 and Gr-1 using a BD Biosciences FACSCalibur equipped with CellQuest software. Analysis of FACS data was performed using WinMDI software. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Columbia University.

RT-PCR

1HAEo cells were grown to confluence and stimulated with 106 CFU/ml heat-killed P. aeruginosa. RNA was prepared using the High Pure RNA Isolation kit (Roche) according to the manufacturer’s instructions. One microgram of total RNA was used as template to generate cDNA using the iScript cDNA Synthesis kit (Bio-Rad). Two microliters of cDNA was used as template to generate cDNA using the QuikChange Site-Directed mutagenesis kit (Stratagene). A Cx43 antisense primer was used to amplify a 156 bp fragment of Cx43, and the Cx43 sense primer was used to amplify a 512 bp fragment of Cx43. The two cell populations were mixed at the ratio of 10:1 (Vybrant DiI:Calcein-AM) and were plated to ensure confluence. After 1 h, 106 CFU/ml P. aeruginosa PA01 were added to selected wells. Monolayers were visualized by confocal microscopy or trypsinized, resuspended in PBS, and analyzed on a FACS Calibur using CellQuest (BD Biosciences) and DOWNF. 4h following bacterial inoculation. After 4 h of infection, mice were euthanized with pentobarbital. Lungs were collected and cell suspensions were subjected to red cell lysis, blocking of nonspecific binding by incubation with 10% normal mouse serum and mouse Fc block (clone 24G2; BD Biosciences), and staining with FITC-conjugated anti-Ly6G/Ly6C (clone RB6-8C5; BD Biosciences) and PE-conjugated anti-CD45 (clone 30-F11; Caltag Laboratories) or appropriate isotype controls. Cells were gated on their forward scatter/side scatter properties and analyzed for expression of both CD45 and Gr-1 using a BD Biosciences FACSCalibur equipped with CellQuest software. Analysis of FACS data was performed using WinMDI software. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Columbia University.

Quantification of intercellular communication

A coculture assay to monitor gap junction communication was performed as previously described (24). Subconfluent monolayers of 1HAEo cells were trypsinized and loaded with calcein-AM or Vybrant DiI as indicated by the manufacturer (Molecular Probes). Calcein-AM is cleaved by cytosolic esterases and becomes exclusively gap junction permeable. Vybrant DiI associates with cell membranes and does not transfer to unlabeled cells. 1HAEo cells expressing Flag-TLR2 were pretreated with 0.02% pluronic acid in MEM. Cells were washed with PBS and incubated at room temperature for 1 h in MEM. Fluor-3/AM fluorescence images were collected using a Zeiss LSM 510 META scanning confocal microscope and analyzed using the ImageJ program. For some experiments, TLR2 siRNA-expressing cells, labeled with Vybrant DiI, were mixed in excess with unlabeled cells expressing the pRS scrambled plasmid. For these experiments, TLR2 small-interfering RNA (siRNA) 1HAEo cells were infected with both pRS-TLR2-1 and pRS-TLR2-2 or a pRS-scramble retrovirus for 18 h in the presence of 1 μg/ml polybrene (Sigma-Aldrich) and selected on puromycin as previously described (19, 25). The pBabe-puro-enhanced GFP plasmid was used to monitor the efficiency of transfection to 293T cells and infection to airway cells. Knockdown of TLR2 and lack of off-target effects of the retroviral infection were verified by Western blotting.

Ca2+ imaging

1HAEo cells were grown to 95% confluence in cover glass chamber slides and loaded for 45 min at room temperature with 2 μM Fluoro-3/AM in the presence of 0.02% pluronnic acid in MEM. Cells were washed with PBS and incubated at room temperature for 1 h in MEM. Fluor-3/AM fluorescence images were collected using a Zeiss LSM 510 META scanning confocal microscope and analyzed using the ImageJ program. For some experiments, TLR2 siRNA-expressing cells, labeled with Vybrant DiI, were mixed in excess with unlabeled cells expressing the pRS scrambled plasmid and allowed to reach >90% confluence before Fluoro-3/AM labeling. Images are shown representative of at least three independent experiments.

CXCL8 ELISA

1HAEo cells were grown to confluence on 96-well plates and weaned from serum overnight. Cells were preincubated with inhibitors for 30 min, stimulated with 106 CFU/ml P. aeruginosa PA01, 15 μg/ml FSC, or 1 μM thapsigargin for 60 min in the presence or absence of inhibitor, and CXCL8 was measured in the supernatants by ELISA according to the manufacturer’s instructions (BD Pharmingen). For kinetic studies, the cells were incubated for the indicated times with P3C, washed with PBS to remove
agonist, and incubated for 3 h in fresh MEM before supernatant collection. Data for each condition were normalized to total protein and plotted as the fold increase over unstimulated control or plotted as the percent of stimulated untreated control or for sextuplicate samples and are representative of at least three independent experiments.

Preparation of triton soluble and insoluble fractions and immunoprecipitations

1HAEo cells were grown in 6-well dishes to confluence and stimulated with $10^8$ CFU/ml heat-killed *P. aeruginosa* for the indicated times. After stimulation, cells were lysed in ice-cold PBS containing 1% Triton X-100, Complete Mini protease inhibitor (Roche), 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride and 100 mM sodium fluoride for 30 min. Cells lysates were centrifuged for 10 min at 13,000 x g and the supernatants were collected as the Triton-soluble fractions. The Triton-insoluble pellets were resuspended by sonication in RIPA (radioimmunoprecipitation assay) buffer (1% Nonidet P-40, Complete Mini protease inhibitor (Roche), 0.25 mM sodium deoxycholate, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 100 mM sodium fluoride). Protein concentration was determined with the Bio-Rad Protein Assay and equal amounts of protein were separated by SDS-PAGE. Den- sitometry was performed using a Molecular Dynamics Model 345 densitometer. Scanner control SI (Molecular Dynamics) was used to capture images and IQMac version 1.2 was used for analysis. Immunoprecipitations and Western blotting were performed as previously described (26).

**NF-κB luciferase assay**

1HAEo cells stably expressing Cx43 wild-type and mutant constructs were grown on 24-well plates to 50% confluence and transiently transfected with a NF-κB-luciferase reporter plasmid using Fugene 6 (Roche). After 24 h, cells were weaned from serum for 16 h and then stimulated with $10^8$ CFU/ml heat-killed PAO1 in MEM for 1 h followed by a 3-h incubation in MEM alone. Lysis and luciferase assays were performed using the reagents and protocol for the dual luciferase reporter assay system (Promega) and analyzed with a luminescence plate reader. Luciferase activity was standardized by protein concentration; data were plotted as fold increase over unstimulated for quadruplicate samples and are representative of at least three independent experiments.

**Statistical analysis**

Statistical significance between groups was evaluated by the Student t test using GraphPad Instat version 3.0 (GraphPad), with the exception of
mouse experiments which used the Mann-Whitney U test. Differences between groups were considered significant at \( p < 0.05 \).

**Results**

**Expression of connexins in airway epithelial cells**

To establish how connexins might be linked to innate immune signaling in airway cells, we first examined the effects of bacterial stimulation on the expression of the major connexins in airway cells. Constitutive expression of Cx26 and Cx43 was suggested by RT-PCR over an 8-h period following bacterial exposure, with substantially less Cx32 observed (Fig. 1A). The distribution of the connexins following bacterial stimulation was also examined as both TLR2 and Cx43 have been previously localized to lipid raft or Triton-insoluble cell fractions in association with a caveolin-1 scaffold (27, 28). Cx43 was found almost exclusively in the Triton-insoluble fraction (Fig. 1B). In addition to Cx43, TLR2 and proteins known to interact with Cx43, namely caveolin-1, zona occludens-1 (ZO-1), and c-Src (29, 30), were also abundant in the fraction enriched for cellular components expected to be organized in lipid rafts (Fig. 1B).

**Ca\(^{2+}\) transients move through gap junctions in airway cells**

To determine whether TLR2 initiated Ca\(^{2+}\) fluxes are communicated through gap junctions, experiments were performed with cells expressing scrambled siRNA, unable to generate Ca\(^{2+}\) fluxes in response to the TLR2 agonist P3C (Fig. 2A), whereas the cells expressing TLR2 siRNA were incapable of responding to this agonist, but responded to thapsigargin (Fig. 2B). By labeling the TLR2 siRNA cells with Vybrant DiI (red) and coculturing them with unlabeled cells that are competent to respond to P3C, the induced Ca\(^{2+}\) wave was observed to spread into adjacent TLR2 siRNA cells (Fig. 2C and Supplementary figure 1). This Ca\(^{2+}\) wave could be communicated either through gap junctions, as we predicted, or by paracrine signaling in which secreted ATP from the stimulated cell activates purinergic receptors on adjacent epithelial cells causing Ca\(^{2+}\) release (31). To test for this possibility, the epithelial cells were pretreated with either a gap channel blocker, 18a-glycyrrhetinic acid (10 \( \mu \)M) (Fig. 2D), or with apyrase (50 U/ml), which hydrolyzes extracellular ATP (Fig. 2E), and changes in Ca\(^{2+}\) were monitored. Ca\(^{2+}\) fluxes were not observed in the TLR2 siRNA cells in the presence of the gap junction blocker, whereas apyrase had no effect, indicating that spread of the Ca\(^{2+}\) transients from cells stimulated with a TLR2 agonist was mediated by gap junctions.

**Regulation of inflammatory signaling through gap junctions**

TLR2 initiated Ca\(^{2+}\) fluxes rapidly stimulate NF-\(\kappa\)B and MAPKs to induce expression of cytokines and chemokines such as CXCL8 (2). We investigated the time course of CXCL8 secretion in HAE\(^{-}\) cells in response to P3C stimulation (Fig. 3A). Consistent with our earlier findings regarding MAPK and NF-\(\kappa\)B activation in expressing scrambled siRNA, initiated within seconds by the TLR2 agonist P3C (Fig. 2A), whereas the cells expressing TLR2 siRNA were incapable of responding to this agonist, but responded to thapsigargin (Fig. 2B). By labeling the TLR2 siRNA cells with Vybrant DiI (red) and coculturing them with unlabeled cells that are competent to respond to P3C, the induced Ca\(^{2+}\) wave was observed to spread into adjacent TLR2 siRNA cells (Fig. 2C and Supplementary figure 1). This Ca\(^{2+}\) wave could be communicated either through gap junctions, as we predicted, or by paracrine signaling in which secreted ATP from the stimulated cell activates purinergic receptors on adjacent epithelial cells causing Ca\(^{2+}\) release (31). To test for this possibility, the epithelial cells were pretreated with either a gap channel blocker, 18a-glycyrrhetinic acid (10 \( \mu \)M) (Fig. 2D), or with apyrase (50 U/ml), which hydrolyzes extracellular ATP (Fig. 2E), and changes in Ca\(^{2+}\) were monitored. Ca\(^{2+}\) fluxes were not observed in the TLR2 siRNA cells in the presence of the gap junction blocker, whereas apyrase had no effect, indicating that spread of the Ca\(^{2+}\) transients from cells stimulated with a TLR2 agonist was mediated by gap junctions.

\footnote{The online version of this article contains supplemental material.}
response to *P. aeruginosa* (32), we observed maximal CXCL8 secretion after 1 h of exposure to P3C that rapidly decreases, suggesting regulation of this inflammatory response.

As the Ca\(^{2+}\) transient generated by TLR2 ligation is itself sufficient to activate CXCL8 secretion (4, 19), we sought to investigate whether these Ca\(^{2+}\) fluxes could elicit proinflammatory responses in adjacent unstimulated cells using gap junction communication. To test this hypothesis, 1HAEo\(^{-}\) cells were pretreated with the gap junction blocker 18α-glycyrrhetinic acid and stimulated with either P3C or thapsigargin for 1 h, and CXCL8 secretion was measured (Fig. 3B). As predicted, blockade of gap junction communication between these cells reduced this chemokine response. Additionally, we found that the same was true when 1HAEo\(^{-}\) cells were stimulated with the physiologically relevant *P. aeruginosa*; CXCL8 secretion was inhibited by the gap junction blockers 18α-glycyrrhetinic acid and carbenoxolone (Fig. 3C). To address the possibility that secreted ATP could elicit Ca\(^{2+}\) fluxes in adjacent cells via purinergic receptors, we performed this experiment in the presence of the nucleotidase apyrase. Inhibition of purinergic signaling did not block CXCL8 secretion in response to *P. aeruginosa*. Taken together, these results suggest that gap junction communication, but not purinergic signaling, contributes to the CXCL8 response to this inhibited pathway.

We wished to further verify the involvement of gap junction communication in the inflammatory response to an inhibited pathogen using a well-documented neonatal model of *P. aeruginosa* infection (2). Mice infected with 10\(^5\) CFU of *P. aeruginosa* PAO1 exhibit a substantial influx of neutrophils into their lung by 4 h after infection. This response was significantly (*p < 0.05, as compared with vehicle-treated PAO1-infected mice*) inhibited by pretreatment of these mice with the gap junction inhibitor 18α-glycyrrhetinic acid (Fig. 3D), which has been previously shown not to diminish neutrophil chemotaxis by itself (33).

**FIGURE 4.** Intercellular communication is inhibited at 4 h following *P. aeruginosa* exposure. A. Communication between 1HAEo\(^{-}\) cells was measured by quantifying the transfer of the gap junction permeable, green fluorescent dye calcein-AM to Vybrant DiI-labeled cells (red) by flow cytometry and plotted as the number of double-labeled cells (*, *p < 0.001, Student’s *t* test). Data are the average of quadruplicate samples and are representative of at least three experiments. B. Cells treated as in A were imaged by confocal microscopy. Images are representative of at least three independent experiments.

Epithelial cells exhibit repeated waves of Ca\(^{2+}\) transients in response to bacterial ligands (4); thus, open gap junctions could facilitate spread of proinflammatory signals throughout the airway epithelium. However, gap junction communication is known to be regulated through intermolecular interactions and phosphorylation (8, 34), and is likely to be subject to similar regulatory mechanisms in airway cells as part of the TLR2 cascade. Gap junctions were open in airway cells in the absence of bacterial stimulation (Fig. 4) and immediately following P3C application (Fig. 2), but by 4 h poststimulation movement of the gap junction permeable dye calcein from cell to cell was significantly inhibited (Fig. 4), suggesting that an autoregulatory cascade activates gap junction closure following the initial response to TLR2 activation, consistent with the kinetics observed for CXCL8 secretion (Fig. 3A).

**FIGURE 5.** TLR2 signaling mediates phosphorylation of Cx43. A, Triton-insoluble lysates of *P. aeruginosa*-stimulated 1HAEo\(^{-}\) cells expressing scrambled or TLR2 siRNA were immunoblotted for active (pY416) and total c-Src. Densitometry was performed on pY416 bands and normalized to total c-Src. Data are presented as the fold increase of pY416 over unstimulated cells. B, c-Src immunoprecipitates (IP) from cell lysates were probed for phosphotyrosine (pTyr) or Cx43. D, Lysates from scramble and TLR2 siRNA-expressing cells were subjected to Western blotting for TLR2, Cx43, Cx26, and β-actin as a control. Data are representative of three independent experiments.

**TLR2-dependent signaling induces phosphorylation of Cx43**

In many types of cells, Cx43 activity is regulated by c-Src phosphorylation (11, 35, 36). To determine whether c-Src is similarly involved in the TLR2-signaling cascade, we first monitored c-Src activation in cells expressing TLR2 following exposure to bacteria. By 1 h poststimulation, there was an increase in c-Src phosphorylation at tyrosine 416, a site associated with kinase activation (37), whereas cells expressing TLR2 siRNA did not show activation of c-Src upon exposure to bacteria (fold increase of 1.6 vs 0.7) (Fig. 5A). TLR2 RNA interference was verified in these samples by Western blotting and no off-target effects were observed for Cx43, Cx26, or β-actin (Fig. 5D).

We next examined whether bacteria stimulate an interaction between c-Src and Cx43 which has been shown to decrease gap
directly involved in amplifying proinflammatory signals initiated three independent experiments. Cells expressing WT Cx43, Student’s t test mutants (STOP). The cells were transiently transfected with an NF-κB reporter construct, exposed to P. aeruginosa and luminescence was measured. The data are represented as the fold increase in NF-κB activity measured at 4 h after stimulation over that of unstimulated cells (*, p < 0.01 compared with cells expressing WT Cx43, Student’s t test). Data are representative of three independent experiments.

Cx43 phosphorylation limits proinflammatory signaling
Experiments were done to establish whether the state of the Cx43 channel is directly involved in the amplification of the epithelial proinflammatory response. The activation of NF-κB was measured in airway cells expressing Cx43 with a mutation in a C-terminal truncation at alanine 257 (STOP). These mutants are unable to close Cx43 channels in response to c-Src (38–40). Airway cells expressing either the Y247F or a C-terminal truncation at alanine 257 was involved in the spread of proinflammatory Ca2+ fluxes in airway cells and to host defense is less well-understood. At least one consequence of the Ca2+ fluxes activated by TLR2 is the amplification of proinflammatory responses to bacteria, by activating adjacent cells through gap junctions.

Increased amounts of intracellular Ca2+ have been associated with the hyperinflammatory state and endogenous activation of NF-κB in airway epithelial cells from cystic fibrosis (CF) patients (44, 45). Although this has been ascribed to elevated stores of endoplasmic reticulum Ca2+ due to repeated bacterial stimulation (46), it may also affect the status of gap junction communication. Chanson et al. (11) suggested that c-Src-associated gap junction regulation was defective in CF cells, although a specific mechanism that links c-Src and defective CF transmembrane conductance regulator activity was not proposed. There are high levels of proinflammatory cytokines in CF airways, particularly TNF-α (47) and ample evidence that links TNF-α, IL-1β, and IFN-γ with decreased gap junction communication (48). Connexin expression in various types of immune cells, monocytes, macrophages, Langerhans cells, microglia, and dendritic cells (6, 22, 49) has been documented and contributes to effective immune signaling. In CF, elevated levels of TNF-α are present, but apparently fail to activate gap junction closure (11). Our data along with that of Chanson et al. (11) and Ribeiro et al. (46, 50) suggest that persistently elevated Ca2+ in CF cells may be linked to the failure of c-Src gating of the channel. Recent work has demonstrated that increases in intracellular Ca2+ are themselves responsible for decreasing gap junction communication and dependent on calmodulin, although the levels of intracellular Ca2+ observed in that particular epithelial cell type (300–700 nM) (51) were much higher than has been observed in airway epithelial cells in response to bacteria (100 nM) (4).

Cx43 channels are gated in airway cells through c-Src phosphorylation of the C termini. Although alterations in Cx43 protein levels have been observed in other inflammatory situations (52, 53), our work and that of others shows that changes in intercellular communication can occur without modulating Cx43 expression but rather through alterations in the phosphorylation state of Cx43 (11, 54). This response was not an immediate consequence of TLR2 ligation, but was somewhat delayed and detected at 4 h following stimulation. Several independent experiments were consistent with this delayed kinetics: the coimmunoprecipitation of Cx43 and c-Src and the demonstration of Cx43 phosphorylation at 4 h leading to the decrease in intercellular communication seen at the same time point. Exactly what signals the recruitment of c-Src to Cx43 is unclear but suggests that there are additional components in the autoregulatory cascade. The overall effect of this gating, however, enables the epithelium to immediately respond to bacterial infection but limits the extent of signaling, shutting off gap junction communication at a time when professional phagocytes should already be recruited and active.

The participation of gap junctions in mucosal defense appears to vary depending upon both the nature of the pathogen and the specific epithelial barrier that is activated. In the experiments presented, we used an extracellular pathogen, P. aeruginosa, that activates airway cells through recognition by TLR2 (19). P. aeruginosa activation of TLR2/Ca2+ transients and NF-κB can be reproduced by superficial stimuli such as the TLR2 agonist P3C or by Ab to a TLR2 coreceptor asialoGM1 (2). The intracellular pathogen Shigella, which targets gastrointestinal epithelial cells,
similarly activates Ca\textsuperscript{2+} fluxes, which amplify the host inflammatory response. However, *Shigella* exploits open gap junctions to facilitate its own cell to cell dissemination (13). Whether this process is specific to the gut epithelium or to *Shigella* is not established, but indicates that cell-cell communication in mucosal cells is an inherent component of the host response to mucosal infection. In contrast to the airway epithelium, TLRs are not accessible on exposed surfaces of gut mucosal cells, preventing consistent activation by commensal flora (55). Thus, it is not surprising that Ca\textsuperscript{2+} fluxes of sufficient amplitude to travel through gap junctions in the gut epithelium are only generated in response to invasive, as opposed to superficial, infection.

The association between innate immune signaling initiated by TLR2 and gap junction communication provides a previously unrecognized mechanism to account for the coordinated behavior of a mucosal surface in response to perceived infection. The components of this signaling cascade, the generation of Ca\textsuperscript{2+} transients and regulation through c-Src association and tyrosine phosphorylation of Cx43 gap junctions, are analogous to that described in detail in other tissues. However, key regulatory components that trigger channel gating remain to be identified, and may be important in diseases of excessive airway inflammation, such as CF. The association between TLR2 activation, generation of Ca\textsuperscript{2+} fluxes, and Cx43 gating provides a potentially useful target for the pharmacological inhibition of excessive mucosal inflammation.

**Disclosures**

The authors have no financial conflict of interest.

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