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Review

Connexins in respiratory and gastrointestinal mucosal immunity

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ABSTRACT

The mucosal lining forms the physical and chemical barrier that protects against pathogens and hostile particles and harbors its own population of bacteria, fungi and archea, known as the microbiota. The immune system controls tolerance of this population of microorganisms that have proven to be beneficial for its host. Keeping its physical integrity and a correct balance with the microbiota, the mucosa preserves its homeostasis and its protective function and maintains host's health. However, in some conditions, pathogens may succeed in breaching mucosal homeostasis and successfully infecting the host. In this review we will discuss the role the mucosa plays in the defense against bacterial pathogens by considering the gap junction protein connexins. We will detail their implication in mucosal homeostasis and upon infection with bacteria in the respiratory and the gastrointestinal tracts.

1. Introduction

The mucosal lining, composed of a stage of epithelial cells on top of a stage of connective tissue bearing the mucosa's vasculature, forms an exchange interface as well as a defense barrier between the exterior environment and the interior milieu of the mammalian body. The mucosa is colonized by a group of microorganisms, named the microbiota that live in symbiosis with host and provide protection against pathogenic microbes [1]. Furthermore, keeping an architectural integrity is a main aspect in maintaining mucosal homeostasis. This integrity is partly regulated by membrane proteins forming structures such as tight junctions, adherens junctions or gap junctions [2]. Gap junctions provide direct cell-to-cell communication between the different cells constituting the epithelial barrier. This communication is mediated by connexin (Cx) channels that ensure direct physical link between cells in a tissue. In addition, Cx channels can also mediate paracrine communication between cells. The modulations of these proteins by pathogens and bacterial toxins has been reviewed by Ceelen and colleagues in 2011 [3]. In this work, we will focus on how these channels can either facilitate infection or, on the contrary, ameliorate host defense and arrest pathogen spreading in the airways' and gut's mucosae. For that purpose, we will detail the mechanisms by which the respiratory and the gastrointestinal mucosae exert their protective role against pathogens according to their respective structures and functions.

2. Connexin- and pannexin-dependent mucosal communication

Direct cell-to-cell communication is essential in coordinating tissue function and homeostasis. This communication, known as gap junction intercellular communication (GJIC), is mediated by gap junction channels that directly connect the cytoplasm of adjacent cells. Gap junction channels are made up of the membrane protein connexins (Cxs). The human Cx family comprises 21 members, with Cx43 being the most frequently expressed in tissues. Six Cxs form a connexon (also called a hemichannel). Connexons of both cells can align and dock to form a gap junction channel that confers a continuous link between the cytoplasm of both cells [4]. They allow for the passage of inorganic ions and of small water soluble molecules; thus, coupling the cells both electrically and metabolically [4]. In addition, connexons were shown to provide communication pathways for small molecules between the intracellular and extracellular environments. Studies over the years have shown that Cx hemichannels communicate with the external cellular environments and have crucial effects on physiological balance [5,6].

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Pannexin (Panx) channels were first described as a family of putative gap junction forming molecules [7]. The human genome codes for 3 Panxs, Panx1 being described as ubiquitously expressed [8]. Further investigation has shown that, despite the fact that Panxs lack homology, they are similar to Cxs at the level of structure and function [9]. However, their ability to form intercellular channels *in vivo* is yet to be demonstrated. The accepted notion is that they form membrane channels known as pannexons [10–12]. Due to the close analogy of Cx and Panx, it is difficult to discuss Cx without evoking the Panx, especially when investigating channel function and formation that cannot clearly differentiate the implication of these two families in observed phenomena [13]. For instance, connexons have been proposed as a putative channel for ATP release (and so have pannexons), and if the characteristics of these two types of channels suggest that pannexons are better candidates, both channels remain studied as ATP-releasing channels [14]. Moreover, due to the similarity in structure and function with Cx, Panx were shown to be blocked by gap junction inhibitors. In this context, assays to study the activity of Cx and Panx1 channels often use the same inhibitors, though at different concentrations [15,16]. Therefore, we will review the relevant roles of Cxs along with Panxs in host–pathogen interaction in the mucosae of the airways and the gastrointestinal tract.

3. Mucosal structures and functions

3.1. The respiratory mucosa

The human upper respiratory tract is formed of a pseudostratified epithelium composed of basal cells, ciliated epithelial cells and non-ciliated, mucus-producing cells that maintain the airway homeostasis [17]. The secreted mucus layer acts as a physical and chemical trap to pathogens and is subjected to continuous motion from the cilia beating; a phenomenon known as mucociliary clearance [18,19]. Recent studies evidenced the presence of a respiratory tract microbiota; however, its role in maintaining homeostasis is still emerging [20]. Being the first line of defense against inhaled hostile particles, the airway epithelium is in constant risk of injury and hence it maintains its integrity by a continuous repair process which eventually restores the well-differentiated epithelium [21,22]. Further downwards in the respiratory tree, the mucosa shows a different morphology and composition. In the alveoli, the epithelial lining is comprised of alveolar type I (ATI) and type II (ATII) epithelial cells. ATI cells cover most of alveolar lumen surface and provide the bulk of the lumen surface for gas exchange, whereas ATII cells secrete surfactant, which is a major determinant of alveolar patency [23]. Alveoli are embedded in a network of capillary endothelial cells allowing gas exchange with the blood circulation. This close proximity with the blood circulation also allows prompt immune cell recruitment upon pathogen infection [24].

3.2. The gastrointestinal mucosa

The stomach and intestines form part of the gastrointestinal tract. The gastric mucosa is composed of a tight epithelial barrier and is covered by a layer made of secreted mucus. The secretion of gastric epithelial cells creates an acidic pH in the lumen, which is an important defense mechanism against pathogens. The epithelial cells are protected from this pH by the production of HCO_3^- in the mucus layer [25].

The intestinal mucosa is formed of a single layer of specialized epithelial cells and of goblet cells [26]. Enterocytes in the small intestine and in the colon are specialized in ion, sugar and amino acid transport while goblet cells produce a protective mucous layer. Depending of the segment of the gut, other cell types, mostly endocrine, can be found. The microbiota plays a beneficial role in maintaining the intestinal homeostasis mainly by preventing the growth of pathogenic microorganisms, aiding in digestion and providing vitamins [27]. Finally, the repair mechanism of the small intestine has been widely described and is crucial to seal the breached area upon injury [26].

4. Mucosal immunity and tolerance

Being at the interface with the outside environment, the mucosal immunity has a complex task of controlling the balance between tolerance of the beneficial microbiota and inducing innate responses against pathogens [1,27]. The immune system recognizes pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs) expressed on host's cells. Upon reaction with PAMPs, PRRs activate specific signaling pathways that lead to antipathogenic effects. PRRs include, among others, toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) [28]. While NLRs are exclusively intracellular PRRs, TLRs are transmembrane proteins that can be expressed either on the cell surface or in intracellular compartments [29]. Depending on their subfamily, TLRs recognize specific extracellular and intracellular PAMPs, and hence activate the appropriate signaling cascade to eliminate the pathogen. For example, surface TLR2, TLR4 and TLR5 recognize extracellular bacterial cell wall glycoproteins, lipopolysaccharide (LPS) and flagellin, respectively; whereas endosomal TLR7, TLR8 and TLR9 recognize internalized nucleic acids [28]. Once TLRs are activated, adaptor proteins are recruited to the TLR's cytoplasmic domain [29]. This recruitment activated signaling pathways, such as mitogen-activated protein kinases (MAPKs), and transcription factors such as nuclear factor (NF)-κB, to initiate an inflammatory response characterized by the production of pro-inflammatory cytokines and chemokines, and by apoptosis [30,31].

5. Connexins in mucosal defense of the respiratory tract

5.1. Ciliary beating and surfactant secretion

A growing body of evidence shows that Cxs and Panxs regulate mucociliary clearance and surfactant secretion by coordinating ciliary beating, airway surface liquid (ASL) volume and mucus secretion. Ciliary beating was shown to be coordinated in a gap junction-dependent mechanism. Indeed, early studies demonstrated that gap junctions regulate beat frequency in airway cells [32]. Later, it was suggested that the propagation of IP_3 through Cx channels mediates Ca^{2+} waves propagation between airway epithelial cells to coordinate ciliary beating [33]. More recent studies suggest another mechanism of Cx- or Panx-mediated coordination of ciliary beating along the epithelium. It was shown that ATP release to the extracellular environment induced Ca^{2+} signaling in epithelial cells due to type-2 purinergic receptors (P2R) activation in a paracrine fashion [34]. Recently, the release of ATP induced by mechanical stress was found to correlate with functional expression of Panx1 channels in differentiated human airway epithelial cells, as well as in murine airway tissues where Panx1 knockout mice showed impaired ATP release [35,36]. Panx1-mediated ATP release was also shown to be involved in regulating ciliary beating and mucin secretion [37].

Efficient mucociliary clearance requires a well-hydrated mucous layer [18]. A recent study conducted on the airway epithelial cell line Calu-3 revealed the importance of Cxs in regulating ASL volume [38]. The inhibition of gap junction-made of Cx43 with mimetic blocking peptides or with pharmacological inhibitors prevented the activation of a signaling network leading to

prostaglandin E_2-dependent regulation of ASL volume increase. These results, which were confirmed in human primary airway epithelial cells after pharmacological inhibition of GJIC [38], suggest that GJIC coordinates a complex signaling cascade which leads to increase in ASL volume.

In alveoli, surfactant secretion by ATII cells is the main determinant for alveolar patency by the exocytosis of lamellar bodies, a type of phospholipid-rich organelles [23]. This exocytosis is triggered by an increase in cytosolic Ca^{2+}. A study conducted on isolated intact rat alveoli tested whether this cytosolic Ca^{2+} increase was mediated by Cx channels [39]. To this end, photo-excited release of caged Ca^{2+} inside the cells was performed. This led to an increase of Ca^{2+} concentration in the cytosol of selected alveoli as well as neighboring ones, and induced ATII cell secretion, even when photo-excited cells were ATI cells. These observations suggest the existence of interalveolar communication involving ATI and ATII cells, which was blocked by Cx43 inhibitory peptides; hence, arguing that interalveolar Ca^{2+} signaling is regulated by GJIC between ATI and ATII cells and mediates ATII surfactant secretion [39].

Altogether, these reports show an important role for Cxs and Panxs in maintaining the respiratory mucosal homeostasis and sustaining mechanisms essential for mucosal defense. In the following paragraph we will discuss how Cxs contribute in pathogen clearing by their interaction with components of the immune system.

5.2. Pathogen-induced airway mucosa innate immune responses

Pseudomonas aeruginosa, a versatile Gram-negative bacterium able to infect damaged tissues or those with reduced immunity, cause generalized inflammation and sepsis [40]. Although *P. aeruginosa* is readily cleared in the airways of healthy subjects, it can cause acute lung infections in intubated patients or chronic lung infections in Cystic Fibrosis (CF) patients [40]. CF, a congenital defect due to mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, is characterized by excessive and destructive airway inflammation [41].

In this context, an *in vitro* study showed that Cx43 expression was decreased in human epithelial cells isolated from nasal polyps exposed to *P. aeruginosa* LPS for 24 h [42]. The authors suggested that reduced GJIC may lead to decreased intercellular passage of signaling molecules important to coordinate cellular functions, such as ciliary beating. Other studies performed on nasal and bronchial airway epithelial cell lines showed that pro-inflammatory molecules such as LPS, TNF-α and lysophosphatidic acid all reduced GJIC within 15 min by a mechanism involving the tyrosine kinase c-Src and its direct interaction with the C-terminus of Cx43 [43,44]. These results indicate that acute closure of gap junction channels by pro-inflammatory mediators precede their disappearance from airway epithelial cells subjected to longer exposure. This rapid channel gating regulation, however, was defective in CF airway epithelial cells due to lack of c-Src activation [44]. Whether LPS and other mediators affected the expression of Cx43 under more chronic conditions was, however, not evaluated in these studies.

A body of evidence described the role of Cxs in mediating efficient inflammatory responses. A study reported that TLR2 activation triggers intracellular Ca^{2+} waves that are transmitted between airway epithelial cells through Cx43 channels [45]. Moreover, cytosolic Ca^{2+} rise activates downstream inflammatory signals, thereby increasing the production and release of interleukin-8 (IL-8), a powerful chemoattractant for neutrophils leading to their recruitment to the infected area. Interestingly, recruitment of neutrophils in the lung of mice infected with *P. aeruginosa* was significantly inhibited by the intraperitoneal administration of a pharmacological blocker of gap junctions [45]. In agreement with other studies [43,46], the authors observed *in vitro* that bacterial stimulation induced tyrosine phosphorylation of Cx43, its association with c-Src and decrease in GJIC 4 h post stimulation, suggesting a delayed negative regulation of gap junction channel activity. In light with these results, GJIC may therefore represent a mechanism to recruit unstimulated cells to secrete pro-inflammatory mediators; this amplification, however, is regulated by a negative feed-back elicited by the pro-inflammatory mediators. In CF airway epithelial cells, this negative feed-back does not occur, which may contribute to the severity of the disease [43,46].

Another level of interaction between Cxs and the inflammatory response to bacteria can be found in the lower respiratory tract. Two pulmonary Cxs, Cx40 and Cx43, have been described in the lung's microvasculature and show opposite expression patterns during acute lung injury [47]. An earlier study showed that Cx40 is decreased after intranasal instillation of LPS from *Escherichia coli* [48]. Although this study failed to reveal an effect of Cx40 deficiency in lung inflammation of $Cx40^{-/-}$ mice, Chadjichristos and colleagues observed enhanced recruitment of neutrophils to the airways after intratracheal instillation of LPS from *P. aeruginosa* in mice lacking Cx40 specifically in endothelial cells [49]. This effect was associated with a decreased expression and activity of ecto-5′-nucleotidase (CD73), an enzyme that hydrolyzes extracellular adenine nucleotides into adenosine. After its production, adenosine activates receptors such as the adenosine receptor A2B. It was shown that upon A2B receptor activation, leukocyte adhesion to endothelial cells was markedly prevented. Interestingly, targeting Cx40 reduced GJIC and CD73 activity, resulting in enhanced adhesion of neutrophils to endothelial cells, suggesting that Cx40-dependent regulation of CD73 modulates leukocyte transmigration across the alveolar endothelium [49].

In contrast to Cx40, which is decreased, intratracheal instillation of *P. aeruginosa* LPS increased Cx43 expression in mouse lung alveolar septa [50]. In this study, $Cx43^{+/-}$ mice showed nearly 50% fewer neutrophils recruited to the alveolar space after induction of lung inflammation. Mice expressing a truncated form of Cx43 lacking most of the C-terminus ($Cx43^{K258stop}$) had increased neutrophil recruitment in response to instillation of LPS [50]. Since gap junctions containing $Cx43^{K258stop}$ have increased open probability and decreased gating [51], then a role for channel function in neutrophil recruitment rather than a channel-independent role for Cx43, is suggested. Consistent with this model, specific Cx43 blocking peptides reduced adhesion of neutrophils to the surface of mouse alveolar and endothelial cell lines [50]. Parthasarathi and colleagues demonstrated a spread of Ca^{2+} signaling through Cx43 gap junctions in alveolar arterioles of the lung capillary bed [52]. Interestingly, Ca^{2+} signaling was involved in the exocytosis from endothelial cells of P-selectin, a molecule promoting leukocytes recruitment on vascular surface [53]. Altogether, these results suggest a balance between the anti-inflammatory Cx40 and pro-inflammatory Cx43, allowing the immune system to limit tissue damages while defending against pathogens.

5.3. Apoptosis and bacterial clearance

Apoptosis has a key role in maintaining homeostasis. On the one hand, apoptosis, in balance with cell proliferation, regulates the different cell populations of the mucosa [31]. On the other hand, apoptotic cells need to be found and cleared; a mechanism that if defected, leads to pathology [54]. In this context, Cx channels were shown to be involved in the spreading of apoptotic signals. As described in HeLa cells transfected with Cx43, Cx40 and Cx37, apoptotic signal induced by the antitumor antibiotic streptonigrin was spread via gap junctions, and apoptosis induction

was correlated with the type of Cxs, Cx37 failing to mediate significant effect [55]. Apoptosis of airway epithelial cells is an important mechanism for bacterial clearance by the host [56], and for the stimulation of tissue repair [54]. This argument is further supported by reports demonstrating that mucosal macrophages induce apoptosis in target cells when bacterial infection persists, as a means of bacterial killing [57]. In this context, we found, in the Calu-3 cells, that Cx43 mediates apoptosis after *P. aeruginosa* infection [58]. Indeed, Cx43 expression was up-regulated in response to *P. aeruginosa* infection, an effect that was not observed in cells infected with a mutant bacterium lacking flagellin, the TLR5 PAMP. Apoptosis was increased in the presence of a JNK inhibitor but the latter effect was prevented by lentiviral expression of a Cx43-specific shRNA. Interestingly, Cx43 expression and apoptosis was dependent on CFTR. Thus, pharmacological inhibition of CFTR reduced the extent of Cx43 expression and induction of apoptosis, an effect that involved enhanced activation of the JNK MAPK. Conversely, correction of the phenotype in a CF airway cell line by adenoviral expression of wild-type CFTR restored normal JNK activity in response to *P. aeruginosa* infection [58]. Thus, abnormal regulation of Cx43 in CF airway epithelial cells may contribute to the reduced apoptosis and bacterial killing that is observed in this disease [59,60].

P. aeruginosa can also counteract and evade the host immune system by producing quorum sensing molecules, such as N-3-oxo-dodecanoyl-L-homoserine lactone C12, to coordinate bacterial behavior and particularly production of virulence factors, depending on bacterial density [61]. Studies conducted in our laboratory have shown that C12 disrupted epithelial gap junctions in non-polarized airway epithelial cells [62]. This was accompanied by cell shrinkage and blebbing, the first signs of apoptosis. Interestingly, these effects of C12 were not observed in polarized epithelial cells cultured at the air–liquid interface. Under these conditions the polarized cells were able to inactivate C12 and protect themselves from apoptosis. These results suggest that loss of airway epithelial cell integrity, which is the case during acute and chronic infection, impairs C12 degradation, and thus defense of the epithelium to the quorum-sensing molecule, providing an additional mechanism for *P. aeruginosa* invasion [62].

The link between Cx43, apoptosis and bacterial killing is, however, not clear. Of note, opening of Panx1 channels in apoptotic cells via cytoplasmic terminal cleavage by caspases was shown to release nucleotides that act as chemoattractants for phagocytes; therefore, providing a mechanism for clearance of dying cells [63]. It would be possible that this apoptotic "find-me" signal mechanism applies to apoptotic airway mucosal cells. Further studies evaluating this possibility could prove useful in the future.

6. Connexins in mucosal defense of the gastrointestinal tract

6.1. Helicobacter pylori infection and gastrointestinal-associated diseases

The Gram-negative pathogen *Helicobacter pylori* is able to adapt to the acid environment of the stomach [64]. Persistent colonization of this pathogen is responsible for gastritis that can develop into gastric ulcers. Apoptosis and cell proliferation are central mechanisms of ulcers, as these lesions are characterized by dead tissue and disorganized repair. Furthermore, *H. pylori*-induced gastritis is a potential risk factor for gastric cancer [64].

H. pylori actively manipulates host tissues and successfully colonizes the gastric mucosa through the activity of several secreted toxins. The vacuolating cytotoxin A (VacA) is a multifunctional pore-forming toxin that disrupts cell polarity and promotes apoptosis of epithelial cells via cytochrome c release from mitochondria [65]. Interestingly, an implication of Cx43 in VacA-induced cell death was found by Radin and colleagues [66]. Using insertional mutagenesis by gene trap and shRNA strategies against Cx43 in human gastric epithelial cells, the authors demonstrated, in viability assays, that inhibition of Cx43 expression resulted in resistance to VacA-induced cell death. Conversely, HeLa cells exogenously expressing Cx43 became more susceptible to VacA, indicating that the level of expression of Cx43 contributed to apoptosis induced by the toxin. Although the precise mechanism remains to be elucidated, VacA-mediated death of gastric epithelial cells through a Cx43-dependent pathway may be particularly important for the persistence of *H. pylori* infection and in the pathogenesis of the associated gastric ulcers. Moreover, the authors speculated that tissue damages and disorganized repair, characterizing this pathological setting, might favor proliferation of cells that exhibit decreased expression of Cx43, and such cells may have increased potential for malignant transformation [66].

Uncontrolled cell proliferation is the basis of carcinogenesis, and gap junctions may control cell growth by maintaining critical signals at equilibrium between connected cells, an ability that is reduced in most tumor cells [67]. Interestingly, decreased GJIC was found in cultured human gastric cancer cells infected with *H. pylori* [68]. In addition, decreased expression of Cx43 was observed after infection with the same bacterium in another human gastric cancer cell type [69], as well as in precancerous gastric lesions of patients colonized by *H. pylori* [70]. In all studies, down-regulation of Cxs was associated with alteration of cell proliferation [68–70]. Finally, in a clinical study, a correlation was found between the occurrence of *H. pylori*-associated gastric cancers and a polymorphism of Cx37 (Cx37 C1019T) [71]. Jing and colleagues reported in the Chinese population that the risk of *H. pylori*-induced gastric cancer increased significantly for CC and CT genotypes compared to TT genotype as compared to patients with chronic superficial gastritis that were chosen as control group [71]. This polymorphism, resulting in the non-conservative amino acid change P319S in the C-terminus of Cx37, has been previously associated with different diseases [72]. The clinical study by Jing and colleagues reinforced the widely observed growth suppressive role of Cx37 [73,74], and extended this role in the context of *H. pylori*-induced gastric cancers. Altogether, there is a relationship between Cxs and *H. pylori*-induced diseases, whereby Cx37 appears to be protective for the host in regard to cancer, while Cx43 seem to be targeted by the bacterium for pathogeny.

6.2. Infection-induced diarrhea

Diarrhea is a pathology accompanied by a profuse loss of water from the intestine, which could cause severe illness, especially in children, and is a common cause of death in developing countries. Water release, triggered in colonic cells by stimuli such as ATP, is a defense mechanism supposed to wipe out pathogens from the gut. As putative ATP-releasing channels, pannexons might be involved in this mechanism. Recently, Panx1 has been identified by immunohistochemistry in epithelial and goblet cells of human colon biopsies [75], but its functional role in the intestinal mucosa still remains unknown.

Excessive water loss and diarrhea can occur in case of severe infection by Gram-negative pathogenic bacteria such as *Shigella flexneri*, *Yersinia enterocolitica*, *Salmonella enterica* serovar Typhimurium and *Enteropathogenic E. coli* (*EPEC*) [76]. Diarrhea induced by *Citrobacter rodentium* is used as a mouse model for *EPEC* infection in humans as this bacterium shares a lot of similarities with *EPEC*. Interestingly, increased Cx43 expression and Cx43 hemichannel activity was observed in the colon of mice infected with *C. rodentium*. Furthermore, mice heterozygous for Cx43 were

protected from diarrhea compared to the wild-type animals [77]. The authors suggested that release of ions through Cx43 hemichannels could create an osmotic gradient for water secretion. Alternatively, excess release of ATP can also contribute to water loss. As demonstrated in rabbit colonic tissues exposed to the supernatant of colonocytes infected with *EPEC*, *EPEC*-induced ATP release from these cells triggered chloride efflux followed by water secretion [78]. Recent observations suggest that *EPEC* infection involves Cx hemichannels since ATP release by polarized human intestinal cells could be inhibited with pharmacological Cx hemichannel blockers [79]. Similar results were obtained in response to *S. flexneri* and *S. Typhimurium* infection.

6.3. Bacterial infection via hemichannel exploitation

S. flexneri and *Y. enterocolitica* exhibit an invasive phenotype by entering into the host cells where they replicate. For that purpose, these bacteria manipulate the cytoskeleton for uptake and for direct spreading from one cell to another [80,81]. In this context, internalization of *Y. enterocolitica* appeared to be facilitated by the hemichannel function of Cx43 when expressed in HeLa cells [82].

The mechanisms underlining *S. flexneri* invasion of intestinal cells are more documented and prove to influence two stages of the process: the contact between the bacteria and the cells before the uptake, and the spreading of internalized bacteria from cell to cell. Romero and colleagues showed that *S. flexneri* is captured via thin filopodia protruding out of the intestinal epithelial cells [83]. These structures, called "nanometer-thin micropodial extensions (NME)", retract upon attachment with the bacteria, bringing them in close contact with the entry site located near the point of filopodia retraction on the cell body. Interestingly, the authors showed that ATP release through Cx26 hemichannels contributed to the control of actin polymerization within NMEs in Cx26-expressing HeLa cells [83]. This mechanism may represent a mean for bacteria to avoid activating the host innate immunity by escaping from extracellular PPRs.

Upon its internalization, *S. flexneri* spreads between cells via host membrane protrusions formed by modifications of actin polymerization. The number of foci of actin polymerization in cell cultures and the number of bacteria spreading from one cell to another were monitored by microscopy in the Caco2/TC7 intestinal cell line and were both found to be increased by an ATP-dependant release via Cx hemichannels, as assessed by gap junction pharmacological blockers [84]. Using blocking antibodies directed against conserved extracellular domains of Cx26, Cx32 and Cx43, hemichannels formed by these Cxs were mostly localized at the basolateral membrane of polarized Caco2/TC7 cells, and specifically implicated in ATP exit during *S. flexneri* infection [85]. Of note, Simpson and colleagues also confirmed that cellular invasion of *S. flexneri* was decreased in Caco2/TC7 cells knockdown for Cx26 [86]. Finally, the specificity of these Cxs, among others, in supporting *S. flexneri* invasion was evaluated by Man and colleagues in HeLa cells exogenously expressing Cx26, Cx30 or Cx31 [87]. The expression of Cx26 markedly enhanced bacterial invasion while Cx30 and Cx31 had no effect. Of note, in the same study, HeLa cells expressing (R143W) Cx26, a deafness-associated mutant form characterized by impaired Cx26 channel activity [88,89], were tested as well, and did not show any enhancement of bacterial invasion, confirming the involvement of Cx26 in *S. flexneri* infection [87].

In contrast with *S. flexneri*, the diarrhea-causing *EPEC* is a moderately invasive pathogen. It must adhere to the target intestinal epithelial cells and then alter cell functions to induce pathogeny [90]. A recent study reported that overexpression of Cx26 in HeLa cells contributed to *EPEC* adhesion [86]. A significant reduction in *EPEC* adherence was observed in HeLa cells expressing the mutant R143W compared with wild-type Cx26. In the same study, similar reduction in *EPEC* adherence was observed in an intestinal cell line treated with interference RNA against Cx26 compared to controls. These *in vitro* results suggest an implication of Cx26 in the control of bacterial adhesion to the target cells by yet unknown mechanisms.

Interestingly, these implications of Cx26 in gastrointestinal infection may explain how the R143WCx26 mutation, previously reported as a common African population-specific recessive mutations associated to deafness [91], could provide a heterozygous advantage in developing countries where gastrointestinal infections are more severe [87].

6.4. S. flexneri-induced dampening of endogenous danger signaling

ATP release through Cxs hemichannels is an important event for invasion of intestinal epithelial cells. However, marked rises in the concentration of extracellular ATP is also a well-known endogenous danger signal that triggers a protective inflammatory host response [92]. In this context, *S. flexneri* has developed counteracting mechanisms for inhibiting ATP release once internalized. Indeed, Puhar and colleagues recently demonstrated that the *S. flexneri* effector IpgD is very rapidly injected into epithelial intestinal cells during invasion [79]. IpgD, which acts as a phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) 4-phosphatase, yielding the rare product PtdIns5P [93], was found to directly inhibit ATP secretion by Cx hemichannels. Similar effects of *S. flexneri*-induced IpgD on inhibition of ATP release was found in HeLa cells expressing Cx26. Finally, using a rabbit model of intestine infection allowing extracellular ATP measurement, the authors showed enhanced ATP exit from IpgD-deficient *S. flexneri*-infected colonic tissues accompanied by severe inflammation and subsequent tissue damage [79]. Of note, Puhar and colleagues also reported that *S. flexneri* induces a brief ATP release before it is inhibited by IpgD. It is tempting to speculate how this early and transient increase of extracellular ATP would be sufficient for the bacteria to sustain their invasion via ATP- and Cx-dependent mechanisms, as discussed above [83,84], just before it serves as an endogenous danger signal.

6.5. Horizontal intestinal epithelial cell activation in response to S. flexneri

Another mechanism for *S. flexneri* to evade the inflammatory response triggered by its host involves the secretion of effector proteins such as Outer Surface Proteins OspF and OspG. These bacterial products are able to inhibit NF-κb and MAPKs, which collectively promote IL-8 mediated inflammation [94–96]. In this context, gap junctions have been involved in the host strategy to fight back this evasion mechanism. Indeed, Kasper and colleagues demonstrated, in the well coupled Caco-2 intestinal cells, that IL-8 was detectable in bystander cells around those that internalized *S. flexneri* [97]. Similar effects were observed with the invasive *Listeria monocytogenes* and *S. Typhimurium*. Since these bacteria inhibited IL-8 response in infected cells, i.e. cells that internalized the bacteria, the release of IL-8 by bystander cells allowed the onset of an inflammatory response at the vicinity of the site of infection. Interestingly, gap junctions were found necessary for cell-to-cell diffusion of signals that activated NF-κB, ERK, JNK and p38 kinases. Although dependent on NLR activation in infected cells, the nature of the exchanged signals between cells was, however, not elucidated. On the other hand, Dolowschiak and colleagues investigated the indirect epithelial activation of intestinal cells infected with *L. monocytogenes in vitro*, by monitoring the synthesis of the chemokine CXCLl-2. They found horizontal epithelial activation that was

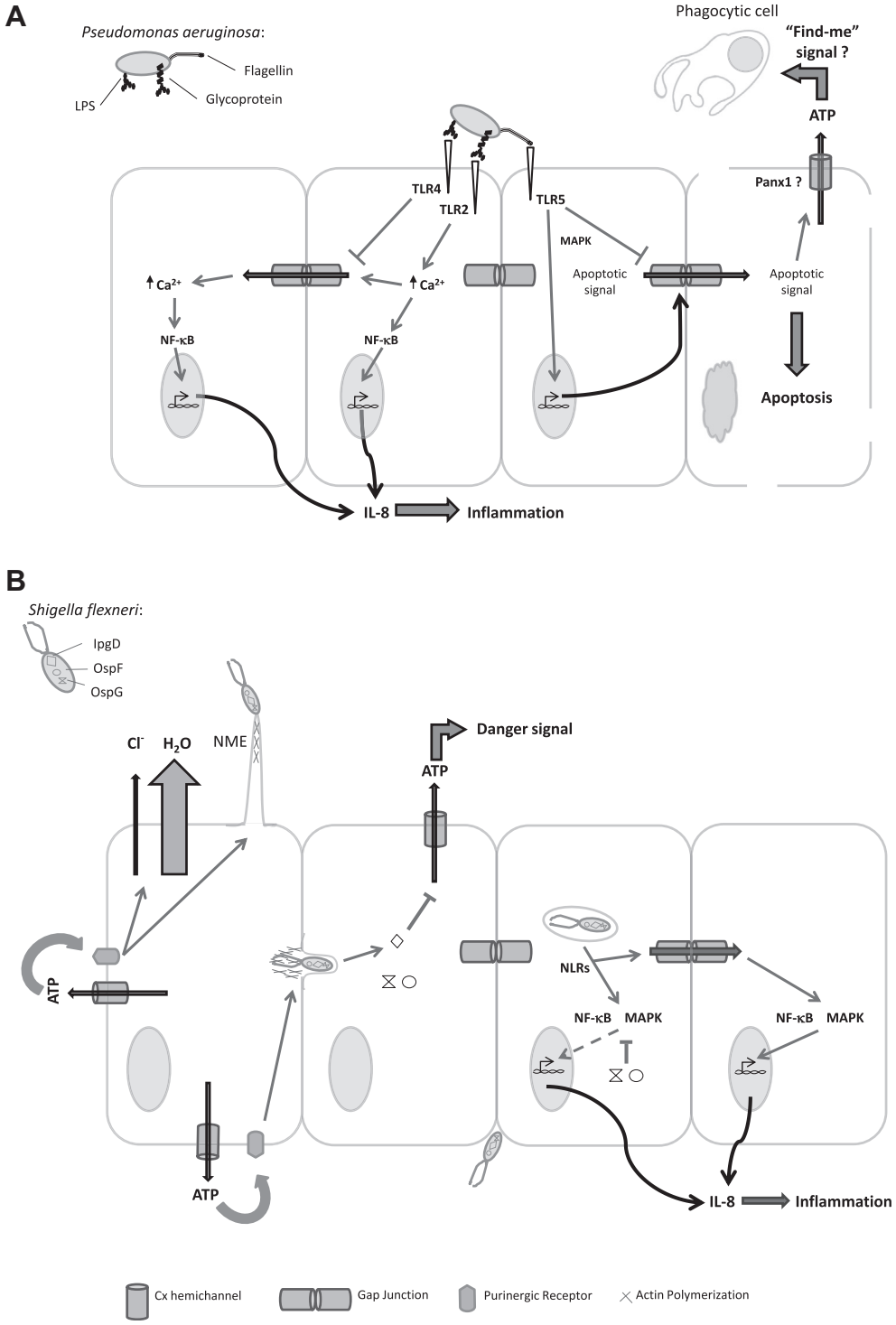

Fig. 1. Putative roles of connexins in host–pathogen interactions during infection of airway epithelial cells with *P. aeruginosa* (A) and of intestinal epithelial cells with *S. flexneri* (B). (A) Recognition of *P. aeruginosa* glycoproteins by TLR2 triggers Ca^{2+} waves in epithelial cells that spread to neighboring cells, activating NF-κB, transcription and release of IL-8, a key chemokine for neutrophil-dominated inflammation. Recognition of *P. aeruginosa* lipopolysaccharide (LPS) by TLR4 may dampen inflammation by reducing Cx43 expression and/or activity. Recognition of *P. aeruginosa* flagellin by TLR5 activates MAPKs, which through multiple regulatory mechanisms enhance the functional expression of Cx43. Enhanced GJIC favors the spread of pro-apoptotic signals to neighboring cells. Apoptosis is accompanied by ATP release, possibly through Panx1 channels, which may contribute to the immune response by attracting phagocytic cells at the site of infection. (B) *S. flexneri*-induced ATP release through Cx hemichannels activates purinergic receptors to trigger outward Cl^- transport and abrupt water secretion, leading to diarrhea. Purinergic intracellular signaling also leads to actin polymerization at NME as well as at the cell membrane to facilitate *S. flexneri* uptake and horizontal infection through cellular protrusions, respectively. Upon direct contact with the cell membrane, *S. flexneri* injects IpgD, OspF and OspG into the infected cell. IpgD inhibits ATP release through Cx hemichannels, possibly to dampen release of the danger signal. OspF and OspG silence the inflammatory response by inhibiting NLR-dependent activation of NF-κB and MAPKs. On the other hand, NLR-dependent signals are transferred via gap junctions to bystander cells. This mechanism activates, in neighboring cells, NF-κB and MAPKs, leading to IL-8-mediated inflammation in the vicinity of infected cells. See text for details and references.

not dependent on gap junctions, as evaluated by chemical inhibitors, but that relied on the diffusion of reactive oxygen intermediates synthesized upon infection [98]. Thus, gap junction-dependent and gap junction-independent mechanisms of horizontal epithelial activation of the immune response exist and may be complementary in counteracting bacterial evasion of the inflammatory defense.

6.6. Commensal-mediated intestinal barrier protection

Commensal bacteria live in symbiosis with the host in resting conditions, and the maintenance of mucosal immunity via PRRs is finely tuned to keep a balance between the microbiota and invading pathogens. In this context, TLR2 activation by commensal bacteria plays a key role in maintaining functional barrier integrity of the intestinal epithelium by suppressing inflammation, establishing tolerance and promoting healing in the presence of mucosal lesion [99]. Ey and colleagues identified a physiological function of TLR2 in the intestinal epithelial barrier maintenance via induction of Cx43 at the transcriptional and post-translational levels [100]. Consistent with this, the activation of TLR2 by its synthetic ligand PCSK was found protective in a chronic model of colitis using MDR1$\alpha^{-/-}$ mice by inhibition of gap junction disruption. The authors also showed that decreased expression of Cx43 in rectal epithelial cells via local RNA interference treatment inhibited the protective role of PCSK against ulcers [100]. Approximately 3–10% of Caucasians are heterozygous for the TLR2 polymorphism R753Q, which has recently been associated with severe ulcerative colitis in patients [101]. Interestingly, this polymorphism, when expressed in an intestinal epithelial cell line, activated the proteasomal pathway leading to excessive Cx43 degradation, thereby impairing TLR2-dependent GJIC. Collectively, these results indicate that Cx43 deficiency results in a defect of TLR2-mediated protection during intestinal injury, suggesting Cx43 exerts a protective role upon TLR2 activation by commensal bacteria in a prophylactic manner in order to heal inflammation-caused injuries.

7. Concluding remarks and perspectives

GJIC coordinates cellular functions essential for sustaining tissue homeostasis but their regulation and contribution in mucosal defenses are not well understood. The roles of Cxs in host–pathogen interactions reviewed here proved to be either beneficial or detrimental during infection, depending on the tissue and the bacterium (Fig. 1). Cxs (and Panxs) are implicated in defense mechanisms such as mucociliary clearance and production of surfactant in the lung, or maintenance of barrier integrity in the intestine. The ability of gap junctions to spread signals between cells was found critical to mobilize bystander cells to mount an inflammatory response following bacterial infection. Interestingly, regulation of gap junction channel activity by pro-inflammatory molecules also appeared to modulate the strength of inflammation, which may avoid unnecessary tissue damage. Finally, apoptosis, a process involved in bacterial clearing, required functional gap junctions. Conversely, pathogens can target gap junctions for destruction but use connexon-mediated ATP release to favor their adhesion, invasion and cell-to-cell spreading, as observed for *S. flexneri* in the intestine. One could notice from the studies reported here that gap junctions mediate protective mechanisms against infection in mucosae, while connexons are more ambivalent. It is worth to note that hemichannel activity has been found to be upregulated by PAMPs in condition in which GJIC was reduced [102,103]. These results suggest an intimate balance between connexon and gap junction activities of a given Cx type upon pathogen recognition, which may be manipulated by pathogens to facilitate host colonization and to contribute to the development of associated diseases.

An additional level of complexity is brought by the existence of Panxs, which are expected to be involved in mucosal defense. These facts may, at first glance, discourage from targeting Cxs and Panxs in prophylaxis and therapy of human bacteria-induced diseases. However, some pharmacological compounds affecting Cxs and Panxs are already used as therapeutic drugs with minimal side effects like for example, Probenecid, a Panx1 channel blocker, used as a gout remedy [13]. There is also much hope for development of efficient RNA interference nucleotides and/or mimetic blocking peptides to target key steps in host–pathogen interactions. Alternatively, the development of strategies to modulate specific molecules that mediate the regulation of GJIC may be proven to be beneficial. In the case of chronic infectious diseases, microbiota therapies, with the aim of restoring a protective flora, may reveal hitherto unexpected consequences on gap junction functions [20].

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