Connexins as therapeutic targets in lung disease

LOSA, Davide, CHANSON, Marc, CRESPIN, Sophie

Abstract

INTRODUCTION: The lung is a mechanically active system exposed to the external environment and is particularly sensitive to injury and inflammation. Studies have identified intercellular communication pathways that promote proper lung function in response to injury and disease. These pathways involve connexins (Cxs) and gap junctional intercellular communication (GJIC). AREAS COVERED IN THIS REVIEW: The functional expression of Cxs in airway epithelium and vasculature, under normal and pathological conditions, is reviewed. Inhibition of GJIC and/or silencing of Cxs have been shown to modulate the course of disease development. Cx-based channels: i) coordinate ciliary beating and fluid transport to promote clearance of particulates, ii) regulate secretion of pulmonary surfactant, in response to deep inhalation by interconnecting type I and type II alveolar epithelial cells, and iii) are key mediators of pro- and anti-inflammatory signalling by the pulmonary endothelium, in order to modulate leukocyte recruitment from the circulation. EXPERT OPINION: Cx-based channels play several central roles in promoting a regulated [...]
Connexins as therapeutic targets in lung disease

Davide Losa, Marc Chanson & Sophie Crespin
Geneva University Hospitals and University of Geneva, Foundation for Medical Research, Laboratory of Clinical Investigation III, Geneva, Switzerland

Introduction: The lung is a mechanically active system exposed to the external environment and is particularly sensitive to injury and inflammation. Studies have identified intercellular communication pathways that promote proper lung function in response to injury and disease. These pathways involve connexins (Cxs) and gap junctional intercellular communication (GJIC).

Areas covered in this review: The functional expression of Cxs in airway epithelium and vasculature, under normal and pathological conditions, is reviewed. Inhibition of GJIC and/or silencing of Cxs have been shown to modulate the course of disease development. Cx-based channels: i) coordinate ciliary beating and fluid transport to promote clearance of particulates, ii) regulate secretion of pulmonary surfactant, in response to deep inhalation by interconnecting type I and type II alveolar epithelial cells, and iii) are key mediators of pro- and anti-inflammatory signalling by the pulmonary endothelium, in order to modulate leukocyte recruitment from the circulation.

Expert opinion: Cx-based channels play several central roles in promoting a regulated inflammatory response and facilitating lung repair, thus enabling the pulmonary epithelium and vasculature to behave as integrated systems. Several pathologies can disrupt the normal communication pathways required for proper lung function, including acute lung injury, asthma, cystic fibrosis, pulmonary fibrosis and cancer.

Keywords: connexins, gap junctions, inflammation, lung disease


1. Introduction

Gas exchange between air and blood takes place in the lung tissue, the largest surface of human body in contact with the environment. This characteristic makes lungs continuously exposed to dust, pollen, chemicals and microbial pathogens, all of which can induce oxidative stress. Protection from these external factors depends on highly effective defence mechanisms which allow clean air to reach the sites of gas exchange.

These mechanisms, mainly represented by mucociliary clearance, surfactant secretion and innate immunity require efficient coordination between the different cell types forming the lung tissues. Connexin-based channels have been shown to be involved in these mechanisms [1], thus importantly contributing to tissue homeostasis and host defence.

1.1 Connexins expressed in lung

Connexins (Cxs) form a family of 20 members in humans [2]. Each connexin contains four transmembrane domains with two extracellular loops, one intracellular loop and two cytosolic extremities. The C-terminal portion is the more variable part of connexins and its length is related to their classification based on the estimated molecular weight of the protein. Thus, in human, the smallest connexin is Cx25 and the largest is Cx62 (respective estimated sizes of 25 and 62 kDa).
Connexins as therapeutic targets in lung disease

**Article highlights.**

- Connexins (Cx) and gap junctional intercellular communication (GJIC) are involved in the homeostasis of all lung compartments.
- Cx expression and GJIC regulation are sensitive to lung injury and disease.
- According to type and/or expression level, Cxs have protective or deleterious effects in lung pathologies.
- The presence of mouse models for Cxs has highlighted the contribution of Cxs in modulating the course of lung injury and inflammation.
- Some of Cxs functions are GJIC-independent, such as hemichannel-mediated transport of ATP to the extracellular space.
- The presence of subtypes of Cxs expressed in diverse compartments such as airway and alveolar epithelial cells, airway endothelial cells and leukocytes adds levels of complexity to target the gap junction proteins in lung disease.
- Cxs are often modulated by drugs used in disease treatments and synthetic blocking peptides of Cxs can reduce lung injury and inflammation in mice.

Formation and function of connexins and gap junctions are subjected to complex regulation, as detailed elsewhere [3], to modulate the number of gap junction channels and their permeability to ions and molecules. Figure 1 shows the topology of Cx43 and its regulatory motifs.

Despite a quasi-ubiquitous distribution, specific patterns of Cx expression are observed depending on organs and tissues. In the respiratory system, several Cxs have been described considering the cell types of the airway tree (Table 1). In human, the upper respiratory epithelium exhibits Cx30, Cx31, Cx32, Cx43 and Cx26 proteins, at least at the foetal level for the latter [4-7]. At the bronchiolar and alveolar levels, Cx26 and Cx43 have been described [7-9]. Interestingly the pattern of Cx expression may be different between species. Thus, unlike what was described in humans, Cx37 may be weakly expressed in airway epithelial cells as well as in alveolar cells of murine models [10-12]. Moreover, Cx32, Cx45 and Cx46 have been described only in murine alveolar cells [13-15].

**1.2 Gap junction channels versus hemichannels**

Various roles have been suggested for Cxs in lung. They are based on Cxs’ ability to form channels allowing the direct communication between the cytoplasm of adjacent cells (gap junctional intercellular communication, GJIC). Briefly, Cxs oligomerize into hexameric structures to form a connexon at the plasma membrane. Usually, connexons between cells in contact dock to form aqueous pores, thus directly connecting their cytoplasm and allowing GJIC [16]. There is however evidence in support [17] and critical [18] of connexons serving physiologic roles. Thus, connexons (referred to as hemichannels) manage a direct access to the extracellular space. Although extending the field covered by this review, the authors would like to introduce pannexins (Px) that present similarities of structure with Cxs. Oligomers of Px (also referred to as hemichannels) do not make gap junctions but form high-conductance plasma membrane channels [19]. Among this family of three members, Px1, initially described in brain [20], has been recently identified in human airway epithelial cell cultures [21]. For clarity, we will use the terminology connexons or pannexons when referring to Cx-made or Px-made hemichannels, respectively.

**1.3 Functions of connexins in lung**

In the airways, ciliary beating, transepithelial fluid movement and hydrated mucus production are parameters finely regulated for an appropriate lung function [22]. It has been speculated that Cx-based intercellular communication may take part in the synchronization of ciliary beating through the propagation of calcium waves between ciliated epithelial cells [23]. Calcium wave propagation may be achieved by the transmission of inositol triphosphate (IP3) from one cell to another via gap junctions [24]. Another mechanism involved the release to the extracellular space of ATP, which in turn would stimulate purinergic receptors and induce calcium signalling in surrounding ciliated epithelial cells [25]. Cxs have also been involved in transepithelial fluid transport and mucus hydration, two important parameters of airway surface liquid (ASL) homeostasis. For instance, GJIC was found to contribute to cystic fibrosis transmembrane conductance regulator (CFTR) activity and fluid secretion in airway epithelial cells in response to adenosine, protease-activated and prostaglandin E2 (PGE2) receptor stimulation [26]. Adenosine is generated from hydrolysis of ATP by ectoenzymes bound to the surface of epithelial cells and leukocytes. The ATP/adenosine ratio appears to be the most important factor for ASL regulation by surface airway epithelial cells [22,27]. Thus, Cxs may provide pathways for the coordinated regulation of mucociliary clearance in the conducting airways (Figure 2A).

In the lower airways, surfactant released by type II alveolar epithelial cells is involved in the reduction of alveolar surface tension and protection against lung injuries and infections [28]. Type I epithelial cells, which represent 90% of alveolar cells and are more subjected to mechanical stress, promote the release of surfactants by type II cells through calcium waves propagation via gap junctions (Figure 2B) [28,29]. It is important to note that transmission of calcium waves from type I to type II cells can also occur via paracrine stimulation of purinergic receptors through ATP release [30,31]. These calcium signals may also help to adjust surfactant production to stimuli such as changes in pulmonary blood pressure [32,33]. Furthermore, GJIC also enables calcium wave propagation from one alveolus to the next [29], as demonstrated by in situ fluorescence microscopy analysis of the intact lung. One implication of interalveolar calcium signalling is that partial lung collapse (atelectasis) or fibrosis can have downstream effects on surfactant secretion by neighbouring alveoli which are otherwise apparently normal by morphological criteria.
The mechanism of ATP release by airway epithelial cells remains poorly understood. It may involve functional connexons, pannexons and/or exocytosis [34,35].

2. Connexins and lung diseases

Connexins and GJIC contribute to lung homeostasis and appear to modulate many aspects that are deregulated in many lung diseases, including acute lung injury (ALI), cystic fibrosis (CF), asthma, pulmonary arterial hypertension (PAH) and cancer. In this second section, we will summarize the different strategies described in these diseases where Cxs may be considered as drug targets.

2.1 Connexins and lung inflammation

Inflammation is a highly regulated defence process characterized by the release of cytokines, chemokines and growth factors and by the transmigration of inflammatory cells, such as neutrophils, monocytes and lymphocytes, from the blood to the affected tissue. Despite the important role of the inflammatory process in repairing tissue injuries, an excessive activation of the immune system or defect in resolution of inflammation has been shown to participate to the pathogenesis of both acute and chronic lung diseases. Since intercellular communication needs to be finely tuned to ensure efficient coordination of cell defence, impaired Cx channel expression and/or regulation may contribute to the abnormal immune response observed in pulmonary diseases [1,36].

2.1.1 Connexins and acute lung inflammation

An acute inflammatory response in ALI can develop in conditions such as sepsis, infection, pneumonia, toxin exposure, trauma and hyperoxia [37]. ALI is characterized by massive infiltration of cells into the lung, formation of protein-rich pulmonary oedema fluid and diffuse alveolar damage, which leads to impaired gas exchange and culminates in respiratory failure.

The rapid diffusion of inflammation from the vascular and alveolar space throughout the entire lung can be responsible of the severity of the diseases. According to Parthasarathi et al. [38], Cx43 has a role in the spread of this inflammatory response. Imaging of the intact perfused lung showed
Connexins as therapeutic targets in lung disease

Table 1. Connexins expressed in lung.

<table>
<thead>
<tr>
<th>Cx</th>
<th>Cell types</th>
<th>Samples</th>
<th>Methods</th>
<th>Species</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26</td>
<td>Airway epithelium (foetal)</td>
<td>Tissue</td>
<td>ISH</td>
<td>Human, Ferret</td>
<td>[7]</td>
</tr>
<tr>
<td></td>
<td>Airway epithelium</td>
<td>Primary cultures</td>
<td>IF, RT-PCR, WB</td>
<td>Human</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>Bronchiolar epithelium</td>
<td>Tissue</td>
<td>IHC</td>
<td>Human</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>Alveolar epithelium</td>
<td>Tissue(foetal)</td>
<td>ISH</td>
<td>Human, Ferret</td>
<td>[7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Primary cultures</td>
<td>RT-PCR</td>
<td>Human</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>IF, WB</td>
<td>Mouse</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Submucosal glands</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx30</td>
<td>Airway epithelium</td>
<td>Primary cultures</td>
<td>IF, RT-PCR</td>
<td>Human</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx30.3</td>
<td>Airway epithelium</td>
<td>Primary cultures</td>
<td>RT-PCR</td>
<td>Human</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx31</td>
<td>Airway epithelium</td>
<td>Primary cultures</td>
<td>IF, RT-PCR, WB</td>
<td>Human</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx31.1</td>
<td>Airway epithelium</td>
<td>Primary cultures</td>
<td>RT-PCR</td>
<td>Human</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx32</td>
<td>Bronchial epithelium</td>
<td>Primary cultures</td>
<td>IHC</td>
<td>Human</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>Alveolar epithelium</td>
<td>Primary cultures</td>
<td>NB</td>
<td>Rat</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>IF</td>
<td>Rat</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>IF, WB</td>
<td>Mouse</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Submucosal glands</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx37</td>
<td>Airway epithelium</td>
<td>Tissue</td>
<td>WB</td>
<td>Mouse</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>Bronchiolar epithelium</td>
<td>Tissue</td>
<td>IF</td>
<td>Rat</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>Alveolar epithelium</td>
<td>Tissue</td>
<td>IHC, WB, ISH</td>
<td>Mouse</td>
<td>[10,12]</td>
</tr>
<tr>
<td></td>
<td>Tracheal smooth muscle cells</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>Bronchiolar smooth muscle cells</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[4]</td>
</tr>
<tr>
<td>Cx40</td>
<td>Alveolar endothelium</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[44]</td>
</tr>
<tr>
<td>Cx43</td>
<td>Airway epithelium</td>
<td>Primary cultures</td>
<td>IF, RT-PCR, WB</td>
<td>Human</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>Bronchial epithelium</td>
<td>Tissue</td>
<td>IHC</td>
<td>Human</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>Alveolar epithelium</td>
<td>Primary cultures</td>
<td>IF, WB</td>
<td>Human</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Primary cultures</td>
<td>IF, WB</td>
<td>Human</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>IF</td>
<td>Rat</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Primary cultures</td>
<td>NB</td>
<td>Rat</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>ISH</td>
<td>Mouse</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>IF, WB</td>
<td>Mouse</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Alveolar endothelium</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[38,40]</td>
</tr>
<tr>
<td></td>
<td>Myoepithelial gland cells</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>Tracheal smooth muscle cells</td>
<td>Tissue</td>
<td>IF</td>
<td>Rat</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>Bronchiolar smooth muscle cells</td>
<td>Tissue</td>
<td>IF</td>
<td>Mouse</td>
<td>[4,103]</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>Primary cultures</td>
<td>NB, RT-PCR, WB</td>
<td>Human</td>
<td>[104,105]</td>
</tr>
<tr>
<td>Cx45</td>
<td>Alveolar epithelium</td>
<td>Tissue</td>
<td>RT-PCR</td>
<td>Mouse</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>Bronchiolar smooth muscle cells</td>
<td>Tissue</td>
<td>Cx45eGFP</td>
<td>Mouse</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>Primary cultures</td>
<td>NB, RT-PCR</td>
<td>Human</td>
<td>[104]</td>
</tr>
<tr>
<td>Cx46</td>
<td>Alveolar epithelium</td>
<td>Tissue</td>
<td>IF, WB</td>
<td>Mouse</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue</td>
<td>IF</td>
<td>Rat</td>
<td>[14]</td>
</tr>
</tbody>
</table>

Cx: Connexin; Cx45eGFP: Mice expressing Cx45 fused with enhanced green fluorescent protein; IF: immunofluorescence; IHC: immunohistochemistry; ISH: in situ hybridization; NB: northern blot; RT-PCR: reverse transcription polymerase chain reaction; WB: western blot.

evidence of calcium waves that propagate along pulmonary vessels. A consequence of this conduction was the expression at the surface of venular endothelial cells of the leukocyte adhesion molecule P-selectin in response to an increase in calcium level in the alveolar capillary bed, thereby promoting leukocyte rolling to the vascular surface. Importantly, this conduction was not observed in mice lacking endothelial Cx43, suggesting a role of Cx43-made gap junction channels as conduits for the spread of pro-inflammatory signals through the lung capillary bed. During the acute phase of lung injury, Cx43 expression increases at the alveolar compartment [39,40]. Moreover, the pro-inflammatory role of Cx43 was confirmed in vivo using Cx43<sup>−/−</sup> mice with reduced Cx expression in the lung [40]. These mice showed an almost 50% decrease in neutrophil recruitment to the alveolar space 24 h after lung inflammation evoked by intratracheal
instillation of *Pseudomonas aeruginosa* lipopolysaccharide (LPS). Conversely, mice expressing a truncated form of Cx43 (Cx43^K258stop mice) exhibited increased neutrophil recruitment in response to LPS instillation [40]. As shown in Figure 1, truncated Cx43 channel at amino acid 257 remains mostly open due to lack of regulatory motifs present in the C-terminus [41]. Together these evidences indicate that during acute lung inflammation Cx43 may therefore represent a pharmacological target to avoid propagation of inflammation in the entire lung and reduce excessive recruitment of leukocytes to the airspace.

During the acute phase of lung inflammatory response, while Cx43 is elevated, Cx40 is oppositely regulated. Cx40 expression decreases in lungs of mouse and rabbit models of ALI [42,43]. This phenotype was associated with increased pulmonary vascular permeability, possibly mediated by overload of intracellular calcium in endothelial cells. Interestingly, at early time (3 – 6 h) of the inflammatory response to intratracheal instillation of LPS, mice with endothelial-specific deletion of Cx40 showed increased recruitment of neutrophils from the blood to the alveolar space [44]. Moreover the lung microvessels of mice with endothelial-deletion of Cx40 showed decreased expression of 5’-ecto-nucleotidase (CD73), the ectoenzyme that hydrolyses extracellular nucleotides to generate adenosine. Endothelial barrier function is modulated by nucleotide signalling during inflammation and several studies indicate that CD73 has protective role in ALI [45-47]. Adenosine produced by CD73 prevents leukocyte adhesion

**Figure 2. Putative roles of GJIC in the airway and alveolar epithelium.** A. In the airways, including bronchioles, diffusion of inositol triphosphate (IP₃) through gap junctions enables the propagation of calcium waves, which help to synchronize ciliary beating to allow directional transport of mucus. Ca²⁺ wave propagation may also activate NF-κB to stimulate the production of pro-inflammatory chemokines. The propagation via gap junctions of cAMP signalling may contribute to CFTR activation and regulation of airway surface liquid (ASL) height. B. The alveolar epithelium is a heterogeneous monolayer consisting of type II cells and type I cells. The alveolus acts as an integrated system where type I cells respond to mechanical stimulation (stretch) with an increase in intracellular calcium which, in turn, is transmitted to type II cells via gap junctions to induce lamellar body fusion and secretion of pulmonary surfactant. Also shown in A and B is the alternative pathway mediated by ATP secretion, via exocytosis and/or hemichannels, and activation of purinergic receptors. ATP can be converted to adenosine (ADO) by the action of ectoenzymes. A₂B/R: adenosine 2b receptor, CFTR: cystic fibrosis transmembranc e conducanc regulator, P2R: type-2 purinergic receptors.
Connexins as therapeutic targets in lung disease

to the endothelium via an intracellular cAMP signalling triggered by stimulation of A2B receptors. Reducing Cx40 expression in vitro with si RNA or antisense nucleotides decreased CD73 expression and activity and increased leukocyte adhesion to a mouse endothelial cell line. Moreover, it was shown that activation of adenosine receptors enhances Cx40-mediated GJIC, enabling the propagation of anti-adhesion signalling between endothelial cells [44]. Thus, Cx40 contributes to anti-inflammatory signalling pathways in lung by preventing neutrophil adhesion to the endothelium.

Cx43- and Cx40-made channels with their specificity in propagating pro- or anti-inflammatory signals between endothelial cells appear as key modulators of acute lung inflammation. The effects of Cx43 and Cx40 genetic manipulation in mice on neutrophils recruitment to the alveolar space during the course of pulmonary inflammation are summarized in Figure 3. Whereas Cx40 delays the adhesion of neutrophils to the endothelial cells at early time of the inflammatory response, Cx43 promotes their transmigration across the endothelial barrier during the acute phase of inflammation. These Cx functions also reflect the opposite regulation of Cx40 and Cx43 expression in acute lung inflammation (Figure 3). Thus, specific inhibition of Cx43 may reduce propagation of pro-inflammatory signalling and enhance Cx40-mediated anti-inflammatory signals in acute lung injury diseases.

2.1.2 Connexins and cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes an apical chloride channel in epithelial cells. A major consequence of CFTR dysfunction in the airway is a lower ASL volume, formation of viscous dehydrated mucus and decreased mucociliary clearance [48]. Thick mucus becomes a nidus for pathogens while slowing the movement of neutrophils that are then unable to clear the infection, leading to a vicious cycle of infection, inflammation and airway tissue injury. The extended inflammatory response to chronic bacterial infection and progressive tissue destruction lead to respiratory failure, which is the major cause of mortality in this disease.

The ASL volume is determined by active transepithelial salt transport, mainly involving absorption of sodium through the amiloride-sensitive epithelial Na+ channel and cAMP-dependent secretion of chloride through CFTR. The latter pathway is stimulated by activation of G protein-coupled receptors (GPCRs), such as adenosine, PGE2 and protease-activated receptors. As mentioned above, activation of these GPCRs also increased GJIC in airway epithelial cells [26]. Inhibition of gap junctions prevents CFTR-mediated chloride secretion and consequent epithelial fluid secretion in a human airway cell line and in primary human airway cell cultures [26]. Thus, functional gap junctions may contribute to the spread of ions (chloride) and second messengers (cAMP) or other cofactors between cells to activate CFTR. Interestingly, previous studies have reported that agents elevating intracellular cAMP were not effective in opening gap junction channels in CF pancreatic epithelial cells [49]. Thus, defective regulation of gap junction channels may represent an additional layer of dysfunction in CF airway disease and contribute to impaired mucus hydration.

Defective regulation of gap junctions may also contribute to the exaggerated inflammatory response characteristic of the CF disease. In airway epithelial cells, Toll-like receptors (TLRs), in particular TLR2, induce immediate calcium dependent signalling that leads to activation of NF-kB and transcriptional activation of pro-inflammatory genes. Martin and Prince [50] showed that Cx43-made gap junction channels transiently amplify this pro-inflammatory signalling by communicating calcium fluxes from stimulated to adjacent non-stimulated cells. However, the latter effect is attenuated in the presence of tumor necrosis factor-α, a cytokine that progressively decreases GJIC by a mechanism dependent on phosphorylation of Cx43 by the tyrosine kinase c-Src [51-53]. This mechanism enables the epithelium to immediately respond to bacterial infection but limits the extent of pro-inflammatory calcium signalling during the course of inflammation. Interestingly, c-Src-associated Cx43 regulation was defective in several airway CF cell lines, resulting in the persistence of gap junction connectivity. This may contribute to the widespread inflammatory response of the CF airway epithelium and elevated levels of IL-8, a potent chemokine for neutrophils [54]. Although several factors are likely to contribute to the pro-inflammatory pathology of CF in lung, these studies identify deregulation of gap junction channels as one of these factors (Figure 2A).

2.1.3 Connexin and asthma

Asthma is a chronic inflammatory disease characterized by airway hyper-responsiveness against environmental antigens that leads to reversible airway obstruction and the consequent recurring episodic attacks of breathlessness, cough, and wheeze [55]. Inflammatory response in asthma consists of infiltration by eosinophils, activated T1/2 lymphocytes and mast cells. Mast cells release inflammatory mediators, which increase mucus secretion, vascular permeability and contraction of bronchial smooth muscle. In this context, it appears that inflammatory cells respond in a coordinated but dysfunctional manner via an array of complex signalling pathways that facilitate communication between immune and structural cells where Cxs could play a role. Interestingly, inhaled carbadoxolone, a well known gap junction blocker of Cx-based channels [56], was shown to prevent allergic airway inflammation in a mouse model of asthma [57]. Inhibition of T1/2 cytokine synthesis, mucus release and eosinophil transmigration by carbadoxolone were associated with a decrease in 5-lipoxygenase expression. However the mechanisms of carbadoxolone action were not investigated in this study. In an allergic airway disease mouse model, Park and collaborators [10] observed the loss of Cx37 expression in bronchiolar airway epithelial cells. The contribution of different Cx types in asthma remains to be investigated.
2.1.4 Connexins in leukocytes

The recruitment of leukocytes, including neutrophils, lymphocytes, macrophages and mast cells to inflamed tissues is a feature of acute and chronic lung inflammation. There is evidence that GJIC is involved in leukocyte extravasation since cellular transmigration across the endothelium was altered in the presence of Cx channel blockers [58-60]. Inhibition of GJIC decreased transendothelial migration of monocytes, increased that of neutrophils but had only modest effects on lymphocytes transmigration. Although it was suggested that gap junctions established between leukocytes and endothelial cells, other studies argued against this idea since neutrophils recovered from inflamed lungs after bronchoalveolar lavages did not show Cx expression or cell coupling [61]. Recently, activated leukocytes were proposed as an extracellular source of ATP through release via Cx37 hemichannels for monocytes and Cx43 hemichannels for neutrophils [62,63], although pannexons may fulfill similar functions [64,65]. The extracellular nucleotide, in turn, regulates leukocyte adhesion to the endothelium as well as endothelial barrier function, including expression of adhesion molecules for leukocytes.

2.2 Connexins and pulmonary arterial hypertension

Pulmonary arterial hypertension is an uncommon disease characterized by increased pulmonary arterial pressure that leads to right-heart failure [68]. Histologically, PAH is associated with a vascular remodelling consisting of proliferation of smooth muscle cells, fibrosis of the intima, hypertrophy...
of the media and increased thickness of the adventice. Progressive endothelial cell dysfunctions lead to an overproduction of vasoconstriction agents among which is endothelin-1. No cure is known at this time. The treatments proposed to attenuate the pulmonary symptoms may consist in anticoagulation oxygen therapy or the use of vasodilator molecules such as endothelin receptor antagonists and PDE-5 inhibitors [69,70]. The last two of these may be related to regulation of Cx43. It was recently showed that Cx43 increased in the pulmonary arterial wall of PAH rat models [70,71]. The effects of endothelin on Cx43 expression have not been investigated yet in other lung cell types. Nevertheless, in heart, endothelin-1 is known to induce an increase in Cx43 expression [72]. In rats exhibiting PAH, a treatment with the PDE-5 inhibitor sildenafil lead to an attenuation of the vascular remodelling associated with decreased Cx43 expression in lung [70]. Thus, acting on Cx43 expression via endothelin receptor antagonists or PDE-5 inhibitors may produce beneficial consequences for PAH. Further work is still needed for understanding of the underlying mechanisms.

2.3 Connexins and lung cancer

Gap junction function has already been suggested as targets for therapeutic strategies in cancer [73]. In this section, we will focus on connexin as potential targets in lung cancer.

Many exogenous agents leading to lung cancer have been associated with an aberrant location or absence of Cxs expressed in lung. Thus, in human samples of non-small cell lung cancer (NSCLC), Cx43 is poorly expressed [6]. This finding was also observed in urethane-induced mouse lung adenomas where both Cx32 and Cx43 were absent [15]. In a rat lung epithelial model, quartz particles, known to induce silicosis and cancer, induce an internalization of Cx43 [74]. It is believed that Cx32 has the potential to regulate lung epithelial cell growth since Cx32-deficient mice are more susceptible to benzene-induced lung toxicity and have a higher incidence of lung tumours [75,76]. From a tissue micro-array of human lung cancer and the study of a large number of human cell lines, Chen et al. [8] showed that the downregulation of Cx26 expression is linked to promoter methylation. Interestingly, when the small cell lung cancer cell line H526 was treated with the differentiation-inducing agent 5-bromodeoxyuridine, the tumour growth rate in nude mice and the ability to grow in soft agar were remarkably reduced. This phenotype was related with an upregulation of Cx26 expression [77]. Nevertheless, the initial assumption suggesting that Cxs would act as tumour suppressor genes has been questioned in several other works (for an extensive review see [78]). Thus, Ito et al. showed in human biopsies that the maintenance of Cx26 expression in squamous cell carcinomas may be related to a higher ability to establish metastasis [79].

Anti-tumour suicide therapy, an emerging strategy against cancer, consists in the introduction of a gene capable of selectively converting a non-toxic prodrug into a cytoplasmic drug in cancer cells. Unfortunately, the efficiency of this strategy is impaired due to the difficulty of introduce the prodrug into a large population of tumour cells. Then, the eradication of the tumours depends on a phenomenon called ‘bystander effect’ leading to the cell death of more tumour cells that the initial number of efficiently transfected cells. Due to their ability to allow direct communication between the cytoplasm of adjacent cells, Cxs have been investigated for their potential to propagate cytotoxic drugs from one tumour cell to another one [80]. In a murine xenotransplant model of human lung cancer, promising results were obtained. The use of a retroviral gene transfer of herpes simplex virus thymidine kinase (HSV-tk) gene followed by systemic ganciclovir application was associated with an improved survival and a reduced tumour growth when tumour cells still expressed Cx43, even with a transfection efficiency that did not exceed 25% of the tumor cell number [81]. Bystander effect has been also observed in radiation therapy. In NSCLC cells submitted to ionizing radiation, an associated treatment with iodide enhanced Cx43 expression and GJIC leading to a potent enhancement of tumour cell killing [82]. Nevertheless, a study by Van Dille et al. [83] showed that the efficiency of HSV-tk/GCV therapy may be challenged by chemoresistance for other treatments used in lung cancer and that bystander effect may be independent of GJIC. Independently of the restoration of efficient GJIC, the expression of Cx may also enhance the effect of drugs on tumour cells [73]. Thus, in the human lung adenocarcinoma cell line A549, Cx32 expression potentiated the effects of the chemotherapeutic agent vinorelbine [84]. The authors showed that Cx32 was related to the decreased expression of multi-drug resistance-1 gene, affecting the ability of the tumour cells to eliminate the chemotoxic drug.

3. Connexins as therapeutic targets

Long-chain alcohol (heptanol, octanol), anesthetics (halothane), 18 alpha glycyrrhetinic acid (tGGA) and carbenoxolone are largely used as blockers of GJIC [56]. In perfused lung, heptanol inhibits the diffusion of endothelial calcium oscillations along the capillaries induced by pressure elevation, which increases the risk of pulmonary oedema in ALI [85]. Although gap junction blockers represent helpful molecular tools to explore the gap junction functions and to alter the movement of molecules through Cx channels, it is quite difficult to consider them as therapeutic drugs due to their potential side effects and the fact that they are not Cx-type-specific.

Small synthetic peptides, corresponding to sequences within extracellular loops of Cxs, were developed as suitable alternative to the traditional pharmacological gap junction blockers [86,87]. Depending of the amino acid sequence, these peptides, called Cx mimetic peptides, may selectively inhibit one type of Cx-made gap junction [88-91]. In contrast to pharmacological gap junction blockers which rapidly inhibit GJIC, peptide action is slower and seems to depend on the
turnover of gap junctions. Although the mechanism of action are unclear and there is no evidence of a direct binding between mimetic peptides and Cxs, it is suggested that the peptides inhibit cell communication by either preventing the formation of new gap junction channels or splitting the interaction of connexons in established gap junctions [92]. In addition, some mimetic peptides were found to block the passage of molecules through connexons and/or connexons [93-95]. Whether channel inhibition would result from a sequence-specific interaction or unspecific steric block of the channel pore remains controversial [96,97].

To define the mechanisms underlying the propagation of pro-inflammatory signals through the lung capillary during ALI, connexin mimetic peptides 43Gap26 and 43Gap27 were used in the intact perfused rat lung. These peptides, respectively, bind the sequences VCYDKSFISHVR and SRPTEKTIFII in extracellular loops 1 and 2 of Cx43 (Figure 1). Pretreatment of rat pulmonary vessels with Cx43 mimetic peptides abolished the spread of calcium waves through gap junctions and the induction of P-selectin expression in endothelial cells [38]. Consistent with this ex vivo finding, Sarieddine et al. [40] investigated the therapeutic potential of anti-Cx43 treatment to modulate neutrophil recruitment in mice during acute lung inflammation evoked by LPS instillation. The intratracheal instillation of 43Gap26 peptides during the course of inflammation efficiently reduced neutrophil recruitment from the blood circulation to the lungs. Interestingly, inhibition of Cx43 did not only attenuate the development of the inflammatory response, but also promoted its resolution when 43Gap26 was administered at peak of inflammation. Inhibition of GJIC by the mimetic peptides was confirmed in vitro, using mouse alveolar epithelial and endothelial cell lines.

Recently, Wang et al. investigated the effects of Cx-based mimetic peptides on hemichannel currents mediated by Px1. 43Gap26 was found to reduce Px channel currents in oocytes, while the effect of 43Gap26 was not detected [96]. The authors suggested that the inhibitory effects of 43Gap27 related are probably indicative of pannexon activity, perhaps resulting from a steric block rather than from a sequence-specific interaction. Although Cx43 connexons open under extreme conditions such as low calcium or hypoxia, pannexons may also represent a target for Cx-based mimetic peptides. One may therefore suggest that the protective effects of mimetic peptides on ALI could be mediated in part by decreased ATP release from pannexons. This idea received support in a murine bleomycin model of ALI where intravenous administration of Px1 mimetic peptide reduced the presence of neutrophils and IL-1β in the bronchoalveolar lavage fluid [98]. These studies suggest that targeting Px could be beneficial during a pulmonary inflammation. Whether this inhibition occurs at the epithelium or leukocyte level is not clear. The effects of Cx43-based mimetic peptides on neutrophil function have been investigated. Although 43Gap26 was found to reduce ATP release and increase the adhesion of neutrophils to the endothelial cells [63], 43Gap26 did not change the number of adhering leukocytes [40]. In addition, neutrophils isolated from Cx43−/− or Cx43K258stop mice did not show alteration in their adherence to endothelial cells. Although additional studies are required to address whether 43Gap27 peptide could affect pannexon function in neutrophils in a non-specific manner, the data indicate that Cx43 mimetic peptides contribute to decrease inflammation in acute or chronic lung diseases characterized by excessive recruitment of neutrophils to the airway.

4. Conclusions

By regulating extracellular and intercellular signalling, controlling the flow of metabolites and restricting the flow of toxic agents, Cxs enable the cells of the lung to act as integrated systems. The appropriate expression and regulation of Cxs have been proposed as possible drug targets in a large range of cancers (for extensive review see [99]). An alteration of their expression is clear in lung cancer, and their restoration may be associated with an improvement of therapy. Nevertheless, the underlying mechanisms still remain to be determined and further investigations should be undertaken to examine their potential. There is considerable evidence from animal and in vitro models that Cxs have the capacity to control lung inflammation. This control occurs at all the compartments of the respiratory tree by regulation of fluid transport and ASL in upper airways, surfactant secretion by airway epithelial cells and leukocyte adhesion to the alveolar capillary bed. In vivo investigations on various mouse models of lung inflammation point to Cx43 as therapeutic targets. The pro-inflammatory role of Cx43 has been observed in other models of tissue injury (see [100] for an extensive discussion).

5. Expert opinion

Although to date no human respiratory disease has been directly attributed to a Cx deficiency or mutation, there is compelling evidence that Cxs and GJIC act to fine-tune lung functions. The targeting of Cxs and GJIC as a therapeutic approach to control lung injury and inflammation will however pose several challenges. Because of the involvement of Cxs in the regulation of cell growth and differentiation, the development of drugs targeting Cxs may have to be associated with investigation of the possible effect on carcinogenesis. However, the approach may be beneficial by affecting either the cause of the pathology or the inflammation that follows it. On the one hand, upmodulation of GJIC between airway epithelial cells may reinforce defence mechanisms by promoting mucociliary clearance via activation of ion transport and ciliary beating. Upmodulation of GJIC may also efficiently stimulate defence against pathogens by enhancing production of pro-inflammatory cytokines, which in turn will recruit more inflammatory cells from the blood.
Connexins as therapeutic targets in lung disease

Table 2. Modulation of connexin expression and GJIC in lung diseases.

<table>
<thead>
<tr>
<th>Models</th>
<th>Tissues/cells</th>
<th>Putative role of GJIC/Cx</th>
<th>Observations for the disease related to GJIC/Cx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute lung inflammation</td>
<td>Mouse Capillary endothelium and alveolar epithelium</td>
<td>Cx43-made channels allow the spread of pro-inflammatory signals [38] and neutrophil recruitment [40]</td>
<td>Increased expression of Cx43</td>
</tr>
<tr>
<td>Mouse Rabbit Capillary endothelium</td>
<td>Cx40 expression contributes to anti-inflammatory signalling pathways by preventing neutrophil adhesion [42-44]</td>
<td>Decreased expression of Cx40</td>
<td></td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Human Airway epithelium Submucosal glands Bronchial epithelium and alveolar epithelium</td>
<td>GJIC may contribute to the ASL volume regulation and mucus hydration [26] Cx43 may mediate pro-inflammatory signals</td>
<td>Increased expression of Cx43 by receptor-dependent activation of CFTR and maintenance of GJIC in CF airway epithelial cells [51-53]</td>
</tr>
<tr>
<td>Asthma and allergy</td>
<td>Mouse Whole lung Bronchiolar epithelium</td>
<td>GJIC may mediate pro-inflammatory signals Cx37 may protect against exacerbation of inflammation</td>
<td>Blockade of gap junctions decreases inflammation [57] Absence of Cx37 expression [10]</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension</td>
<td>Rat Pulmonary artery</td>
<td>GJIC may mediate pro-inflammatory signals</td>
<td>Increased Cx43 expression [70,72,79]</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Human Alveolar epithelium</td>
<td>Cx26 expression may favour cancer cell mobility and metastasis [71]</td>
<td>Decrease expression of Cx43 [6] and Cx26 [8] Squamous cell carcinomas exhibit Cx26 expression as well as the metastatic cells detected in lymph nodes [71]</td>
</tr>
<tr>
<td></td>
<td>Cell line H526</td>
<td>Cx26 expression may be related to a decreased cancer cell growth [77]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell line A549</td>
<td>Cx32 expression potentiates the effects of the chemotherapeutic agent vinorelbine [84]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse Alveolar epithelium</td>
<td>Cx32 may regulate epithelial cell growth Maintenance of Cx43 expression is related to a more efficient HSV-tk/GCV therapy [81]</td>
<td>Absence of Cx43 and Cx32 [15] Cx32-deficient mice present an increased incidence of lung tumors [75,76]</td>
</tr>
</tbody>
</table>

GCV: ganciclovir; HSV-tk: herpes simplex virus thymidine kinase. Please refer to the text for additional references.

circulation. This will also be achieved by enhanced production of surfactants by alveolar type II epithelial cells and increased expression of adhesion molecules for neutrophils by alveolar endothelial cells. On the other hand, downregulation of GJIC may represent a mechanism to reduce the propagation of inflammation by decreasing cytokine/chemokine production and neutrophil transmigration.

The major difficulty with strategies using gap junction modulators is to run the risk of having an adverse effect on processes where the same Cx type have beneficial or deleterious effects on lung functions according to their regulation and/or the lung regions they are expressed. For example, targeting Cx43 may reduce inflammation in the respiratory portion of the lungs but promote infection in the upper airways. Another important point to consider is that ‘selective’ GJIC results from the different types of Cx expressed. The alveolar endothelium, which expresses at least Cx40 and Cx43, is a typical example where the ratio of their level of expression favours either a pro- or anti-inflammatory response along the capillary bed. Table 2 summarizes the known relationships between lung diseases and Cx changes. It is obvious that more knowledge on the functional role of each Cx type expressed in specific regions of the lungs is needed. This may require generation of new mouse models with cell-specific deletion and/or expression of lung Cxs such as Cre-Lox strategies. Because the pattern of Cx expression may differ among species, the use of well-defined primary cultures of human airway/alveolar epithelial and alveolar endothelial cells is much needed. New promising technology for gene editing, such as, for example, the use of zinc finger nucleases [101], may also represent potent tools to target Cx gene at specific sites in the genome of cells in primary cultures. Another approach to
modulate Cx expression would be to take advantage of non-coding RNAs (microRNAs). For instance, Cx43 has been shown to be targeted by specific microRNAs and more are likely to be discovered in coming years for other Cx types. Thus, upmodulation or downmodulation of microRNA functions may provide another potential therapeutic window. However, these strategies will have to deal with target specificity and the method of administration.

In vivo evidence supports the use of mimetic peptides to inhibit Cx43 to attenuate the inflammatory response in lung. Attractive advantages of the Cx-specific peptides are that the inhibitory effect is reversible and that toxic effect, as far it has been investigated, seems to be avoided. A similar strategy could be used to develop peptides that enhance Cx function. On the other hand, these peptides are expected to have a short half-life due to the rapid degradation. Thus, chemical modifications are necessary to enhance the bioavailability, specificity and pharmacological efficacy of the peptides. It may prove in the end that identifying molecules that target subclasses of Cxs and using a combination of these molecules will provide a more fruitful approach to target lung injury and inflammation. Continuing to identify specific roles for specific Cxs will provide an important foundation to determine whether targeting these intercellular communication pathways represents a feasible approach to the treatment of lung disease.

Declaration of interest

The authors declare no conflict of interest.

The work done in the author’s laboratory, part of which is reviewed in this paper, was supported by a grant from the Swiss National Science Foundation to M Chanson.

S Crespin is supported by Vaincre la Mucoviscidose and D Losa is supported by the Italian Cystic Fibrosis Research Foundation (grant FFC#19/2009, with the contribution of FFC delegation “La Bottega delle Donne”).

Bibliography

Papers of special note have been highlighted as either interest (●) or of considerable interest (★★) to readers.

8. The first paper, to our knowledge, that shows the drastic reorganization of gap junctions and of Cx26 during lung development.
21. Ransford GA, Fregien N, Qiu F, et al. Pannexin 1 contributes to ATP release in

999
Connexins as therapeutic targets in lung disease

* This paper demonstrates the apical expression of PtX1 in differentiated airway epithelial cells and the contribution of the hemichannels to ATP release.

- 30. Isakson BE, Evans WH, Boitano S.

** A classic paper showing the mechanism of calcium wave propagation in airway epithelial cells by gap junctional diffusion of inositol trisphosphate.

- 25. Homolya L, Steinberg TH, Boucher RC.
- 27. Andreeva AV, Kutuzov MA,
- 28. Lazarowski ER, Boucher RC.
- 29. Fahy JV, Dickey BF.

** Along with the work in [29-32], this manuscript highlights the role of Cx43 in mediating calcium waves. The paper shows that Cx43-mad gap junctions contribute to endothelial cell expression of adhesion molecules for leukocytes.


** To our knowledge, this is the first manuscript evaluating in vivo the contribution of Cx43 in acute lung inflammation. The work also shows the potential of a Cx blocking peptide in attenuating lung inflammation.


** An interesting paper linking Cx43 regulation to secretion of pro-inflammatory mediators in CFTR-expressing airway epithelial cells.


* **This paper, which initiated important work cited in [44,46,47] and [63], demonstrates in CD73-$^-$ mice the critical role of CD73 as a mediator of vascular leakage in the lung.

A paper reporting defective regulation of CaX by pro-inflammatory mediators in cystic fibrosis airway epithelial cells.


Murphy DM, O’Byrne PM. Recent advances in the pathophysiology of asthma. Chest 2010;137:1417-26.


This paper is an example of how CaX function can modulate the course of an inflammatory disease.


Connexins as therapeutic targets in lung disease

- A recent overview of the mimetic peptides that have been shown to modulate Cx-made channels.
- Bodendiek SB, Raman G. Connexin modulators and their potential targets under the magnifying glass. Curr Med Chem 2010;17:4191-230
- A detailed review of the different classes of pharmacological inhibitors and/or activators of Cx channels.

Affiliation
Davide Losa, Marc Chanson† PhD & Sophie Crespin PhD
†Author for correspondence
Geneva University Hospitals and University of Geneva, Foundation for Medical Research, Laboratory of Clinical Investigation III, 64 Avenue de la Roseraie, 1211 Geneva, Switzerland
Tel: +1 4122 37 24 611; Fax: +1 4122 22 34 75 979; E-mail: Marc.Chanson@hcuge.ch