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Review article

Pulmonary surfactant as a versatile biomaterial to fight COVID-19

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ABSTRACT

The COVID-19 pandemic has wielded an enormous pressure on global health care systems, economics and politics. Ongoing vaccination campaigns effectively attenuate viral spreading, leading to a reduction of infected individuals, hospitalizations and mortality. Nevertheless, the development of safe and effective vaccines as well as their global deployment is time-consuming and challenging. In addition, such preventive measures have no effect on already infected individuals and can show reduced efficacy against SARS-CoV-2 variants that escape vaccine-induced host immune responses. Therefore, it is crucial to continue the development of specific COVID-19 targeting therapeutics, including small molecular drugs, antibodies and nucleic acids. However, despite clear advantages of local drug delivery to the lung, inhalation therapy of such antivirals remains difficult. This review aims to highlight the potential of pulmonary surfactant (PS) in the treatment of COVID-19. Since SARS-CoV-2 infection can progress to COVID-19-related acute respiratory distress syndrome (CARDS), which is associated with PS deficiency and inflammation, replacement therapy with exogenous surfactant can be considered to counter lung dysfunction. In addition, due to its surface-active properties and membrane-interacting potential, PS can be repurposed to enhance drug spreading along the respiratory epithelium and to promote intracellular drug delivery. By merging these beneficial features, PS can be regarded as a versatile biomaterial to combat respiratory infections, in particular COVID-19.

1. Introduction

In December 2019, the Chinese authorities identified severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) as a new emerging β-coronavirus, capable of infecting humans and causing respiratory pathology. Infection with SARS-CoV-2 can result in the development of coronavirus disease-19 (COVID-19), which poses a serious threat to global public health, echoed by an estimated global mortality rate of more than four million people, as reported by the World Health Organization (WHO) [1]. Since its emergence, SARS-CoV-2 has rapidly spread across countries by which, as of March 2020, the WHO officially declared the outbreak as a pandemic. This health crisis led to worldwide efforts to combat COVID-19 by directly targeting SARS-CoV-2 infection (*e.g.* with antivirals), symptomatic treatment (*e.g. via* steroids), as well as through the optimization and standardization of supportive care management such as prone positioning and oxygenation [2–4]. All these efforts have the goal to prevent the development of respiratory failure, the most common cause of COVID-19 mortality [5]. Importantly, global vaccination campaigns are currently ongoing and will further progress in the coming months, leading to a reduction of infections, hospitalizations and mortality [6]. However, the availability of both preventive measures as well as (combination) therapies for already infected patients might be most effective. Therefore, in response to the urgency to fight the escalating COVID-19 pandemic, many existing drugs have been screened with the aim to identify compounds that could be successfully repurposed, since such an approach is more time- and cost-efficient and is correlated to higher success rates compared to the development of novel anti-SARS-CoV-2 drugs [7–10]. A variety of compounds from diverse drug classes including antivirals (*e.g.* remdesivir, favipiravir and lopinavir/ritonavir), antiparasitics (*e.g.* chloroquine and hydroxychloroquine), monoclonal antibodies (*e.g.* tocilizumab) and steroids (*e.g.* dexamethasone) have been investigated in (pre)-clinical trials [11]. In this regard, the broad-spectrum nucleotide mimic remdesivir is approved for emergency use as a post-infection treatment for COVID-19 in around 50 countries [12,13]. Next to this, the WHO strongly recommends the use of dexamethasone in severe COVID-19 patients that require oxygenation or mechanical ventilation [14]. In addition, many groups have investigated the potential of combining different

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(repurposed) drugs to achieve improved therapeutic outcomes. In this aspect, remdesivir is often combined with anti-inflammatory drugs or immunomodulators to both target SARS-CoV-2 as well as inflammatory pathways. This led to the approval of remdesivir and the immunomodulator baricitinib for emergency use by the Food and Drug Administration (FDA). Similarly, also combination therapies of casirivimab and imdevimab as well as bamlanivimab and estesevimab (*i.e.* monoclonal antibodies (mAbs) that target the spike protein of SARS-CoV-2) were granted emergency use approval by the FDA [15].

However, apart from this, advancements in the development of SARS-CoV-2-specific therapeutics are limited [16]. This can in part be explained by antiviral drug candidates often having limited influence on critical parameters such as the need for oxygenation or mortality [17]. However, the development of such specific antivirals as an add-on to vaccination is crucial, especially for already infected patients, individuals who are not yet vaccinated or in cases where vaccination does not provide sufficient protection (*e.g.* upon the emergence of SARS-CoV-2 variants) or causes adverse events (*e.g.* anaphylaxis and cerebral thrombosis) [18]. In this context, Seley-Radtke, Roche and Altea are developing a nucleotide analog (AT527) to directly target SARS-CoV-2 infection after oral administration, which is currently evaluated in a phase III clinical trial (*i.e.* the MORNINGSKY trial). The aim is to administer this antiviral drug candidate to both non-hospitalized and hospitalized patients, as well as to prevent infection in people who experienced a high-risk contact [19]. Next to this, two oral antiviral drugs by Merck and Pfizer (*i.e.* molnupiravir and paxlovid, respectively) are currently under clinical development. Here, molnupiravir was initially developed to treat influenza, while paxlovid is a SARS-CoV-2-specific drug. Both candidates already showed to significantly reduce the number of hospitalizations and deaths of COVID-19 infected patients [20].

Infection with SARS-CoV-2 occurs through binding of the spike protein on the outer shell of the virus to the angiotensin-converting enzyme-2 (ACE-2) receptor, expressed on a variety of host cell types throughout the respiratory system. In particular, there is a significant expression of the ACE-2 receptor on type II pneumocytes, the alveolar epithelial cell type responsible for the production and secretion of PS [21–23]. In severe cases, infection with SARS-CoV-2 leads to the development of CARDS, associated with excessive inflammation, respiratory failure and potentially death [24–27]. The administration of exogenous surfactants to patients suffering from both neonatal (NRDS) and acute respiratory distress syndrome (ARDS) has been widely investigated. This so-called 'surfactant replacement therapy' (SRT) is currently the standard-of-care to reduce mortality in premature infants with surfactant-deficient lungs [28]. Although clinical trials have failed to demonstrate improved survival in ARDS [24,29,30], the current COVID-19 pandemic has invigorated the use of exogenous surfactants to treat CARDS, resulting in several ongoing clinical trials [31–35]. In addition to this well-documented application, this review additionally aims to highlight two distinct modes of action of PS, which could prove highly beneficial in the fight against COVID-19. First, due to its surface-spreading properties, PS can be used as a drug delivery vehicle to achieve improved spreading of COVID-19-targeting drugs along the respiratory surface, as well as to support drug delivery into more distal regions such as the alveolar spaces [2,8,36,37]. In addition, formulating drugs inside PS liposomal carriers can hitchhike down the endogenous surfactant recycling pathway to target specific cell types such as type II pneumocytes or alveolar macrophages (AMs) [38–40]. Secondly, besides improving the extracellular availability of various therapeutics in the lung, PS has the newfound potential to promote the intracellular delivery of membrane-impermeable drugs. In particular, inhalation therapy with small interfering RNA (siRNA) is a promising approach to address acute respiratory viral infections, both by targeting viral proteins responsible for infectivity or replication, as well as host-related proteins that play a crucial role in viral infection or disease severity [41,42]. To be functional, siRNA molecules require cytosolic delivery in the target cell of interest, for which they are typically encapsulated into nanoparticles (*i.e.* nanomedicines). Unfortunately, current state-of-the-art siRNA nanomedicines have difficulties in overcoming the many extra- and intracellular barriers upon topical administration to the lung, leading to inefficient cytosolic delivery [43,44]. In this regard, it was recently disclosed by Raemdonck and *co*-workers that exogenous PS has the unexpected property of promoting cytosolic siRNA delivery by polymeric nanomedicines [45–50]. As such, exogenous PS can be regarded as a multifaceted biomaterial, enabling both direct treatment of COVID-19-related lung dysfunction as well as improved pulmonary delivery of small molecular- and macromolecular drugs.

2. Severe acute respiratory syndrome coronavirus-2

Coronaviruses belong to the family of Coronaviridae, which is further subdivided in four genera (α, β, γ, δ) [51,52]. They are enveloped, single-stranded (ss) RNA viruses with a genome ranging from 26 to 32 kilobases (kb) [53,54]. The ~30 kb long genome of SARS-CoV-2 encodes four structural proteins including the nucleocapsid (N), spike (S), membrane (M) and envelope (E) proteins [41] and a variety of non-structural proteins such as RNA-dependent RNA polymerase and helicase [55]. The spike protein is responsible for the remarkable morphology shared by Coronaviridae, typified by the projection of crown-like structures on their surface [54,55].

Life-threatening lower respiratory tract infections were reported for severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1) in 2003 and Middle Eastern respiratory syndrome coronavirus (MERS-CoV) in 2012 [56,57]. Since December 2019, SARS-CoV-2, the third virus of the β-coronavirus group, is responsible for the current pandemic [55].

As briefly mentioned above, infection with SARS-CoV-2 occurs through binding of the viral spike protein to the ACE-2 receptor, mainly expressed in the pulmonary system on endothelial cells and type II pneumocytes. Of note, the ACE-2 receptor is also expressed in other organs such as the intestine, heart and kidney, explaining the extrapulmonary symptoms such as diarrhea, nausea, cardiac injury and acute kidney injury [51,58,59]. As shown in Fig. 1, viral entry occurs *via* direct fusion as well as *via* endocytic entry routes. The former mechanism of entry requires initial spike protein priming by a host serine protease, transmembrane protease serine 2 (TMPRSS2), which cleaves the protein into two subunits, S1 and S2. In the case of endocytic entry, the spike protein is activated by cathepsin B and L, two cysteine proteases present in the endosomal compartment, followed by fusion between the viral envelope and the endosomal membrane. In this way, SARS-CoV-2 can also infect host cells that lack TMPRSS2 [21,22,]. Once the SARS-CoV-2 genome enters the cytosol, it is transcribed by the viral RNA-dependent RNA polymerase and translated into viral proteins by host cell ribosomes. Finally, mature virions are released *via* exocytosis, where they can further infect surrounding cells [23].

Despite the complete pathophysiological pattern of COVID-19 remaining elusive, infection results in a broad spectrum of clinical manifestations, ranging from asymptomatic to life-threatening. Most common features of moderate COVID-19 include fever, cough, headache, fatigue, myalgia and shortness of breath [53]. Especially older patients and patients with co-morbidities such as obesity, diabetes and cardiovascular disease are at risk to develop respiratory failure with differing levels of hypoxia and diffuse lung infiltrates, associated with multi-organ failure (which necessitates hospitalization in the intensive care unit (ICU) and mechanical ventilation) and a high mortality rate [2,51,54,58,60]. Of note, an increasing number of reports describe persistent complications post-infection that can last more than 4 weeks since the onset of symptoms [61–63].

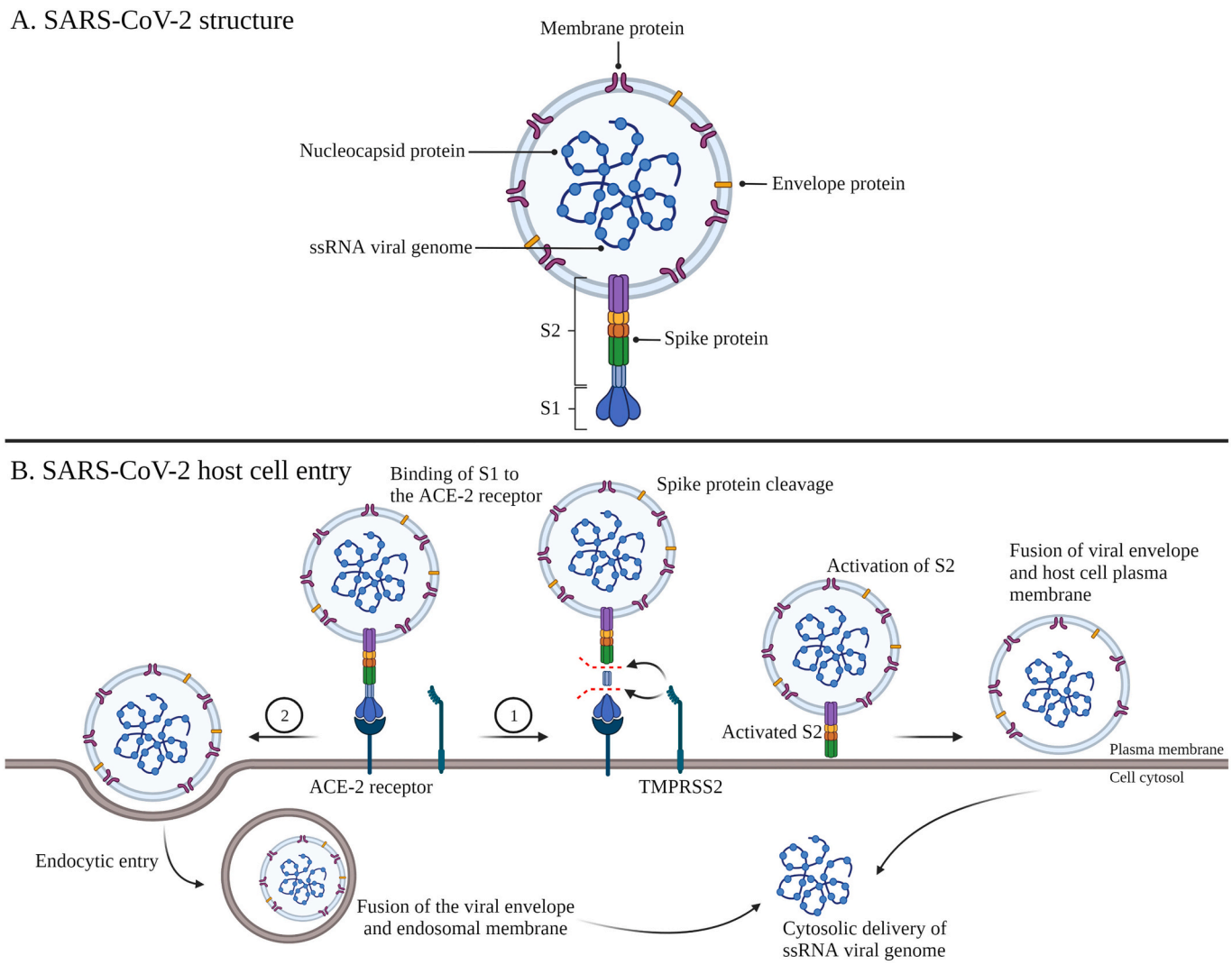

Fig. 1. SARS-CoV-2 structure and host cell entry mechanisms. The single stranded (ss) RNA viral genome encodes four structural proteins including the nucleocapsid, membrane, envelope and spike protein **(A)**. Binding of S1 of the viral spike protein to the ACE-2 receptor on host cells induces 1) spike protein cleavage by TMPRSS2, followed by activation of S2 and viral internalization *via* direct fusion of the viral envelope and the host cell plasma membrane or 2) viral internalization *via* endocytic entry, followed by fusion between the viral envelope and the endosomal membrane **(B)**. Abbreviations: SARS-CoV-2; severe acute respiratory syndrome coronavirus-2, ACE-2; angiotensin-converting enzyme-2, TMPRSS2; transmembrane protease serine 2, S1; subunit 1, S2; subunit 2. Adapted from 'Mechanisms of SARS-CoV-2 Viral Entry', by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

3. Pulmonary surfactant

3.1. Endogenous pulmonary surfactant: Composition and function

PS is a surface-active biomaterial present at the thin layer of fluid that lines the alveolar epithelial surface, where it lowers the surface tension upon the formation of surfactant films at the air-liquid interface (Fig. 2A) [64]. These interfacial films prevent alveolar collapse during expiration and thereby secure proper gas exchange [2,65–67]. The presence of PS is crucial for pulmonary homeostasis, as its absence, deficiency or inactivation is correlated with severe pulmonary diseases [66,68]. As shown in Fig. 2B, PS is mainly composed of lipids (~90 wt%) with dipalmitoylphosphatidylcholine (DPPC), a saturated phospholipid, being most abundant (~40 wt%). In addition, anionic lipids (~15 wt%) such as phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) and neutral lipids such as cholesterol (~8 wt%) contribute to this lipid fraction [65,67]. The remaining 10 wt% consists of four highly specialized surfactant proteins (SPs), which can be divided in two groups based on their structural, physicochemical and functional properties, *i.e.* the large and hydrophilic proteins SP-A (~6 wt%) and SP-D (~1.5 wt%) and the smaller, hydrophobic proteins SP-B (~1 wt%) and SP-C (~1.5 wt%).

PS is synthetized by type II pneumocytes and stored in highly packed intracellular organelles called lamellar bodies (LBs), which have a spherical shape and comprise concentrically organized membranes. Subsequently, upon fusion of the LBs with the plasma membrane, PS is secreted into the alveolar lumen where it partly reorganizes into tubular myelin. Tubular myelin consists of multi-layered lipid-protein structures in the liquid phase, stabilized by SP-A. The presence of SP-B and SP-C subsequently induces the adsorption of these structures onto the interface (Fig. 3) [69]. Upon expiration, unsaturated lipids and cholesterol are excluded from these interfacial films (*i.e.* the squeeze-out model), resulting in the formation of a rigid DPPC monolayer that reduces the surface tension to near 0 N/m (Fig. 2C) [2].

About 65% of PS components (*i.e.* 'used' surfactant) is reutilized *via* recycling mechanisms by type II pneumocytes [70]. As depicted on Fig. 3, after binding of SP-A to the P63/CKAP4 receptor expressed on these cells [71,72], surfactant lipid- and protein complexes (mainly phospholipids and SP-A) are taken up *via* receptor-mediated endocytosis. While SP-A is secreted into the extracellular space *via* recycling

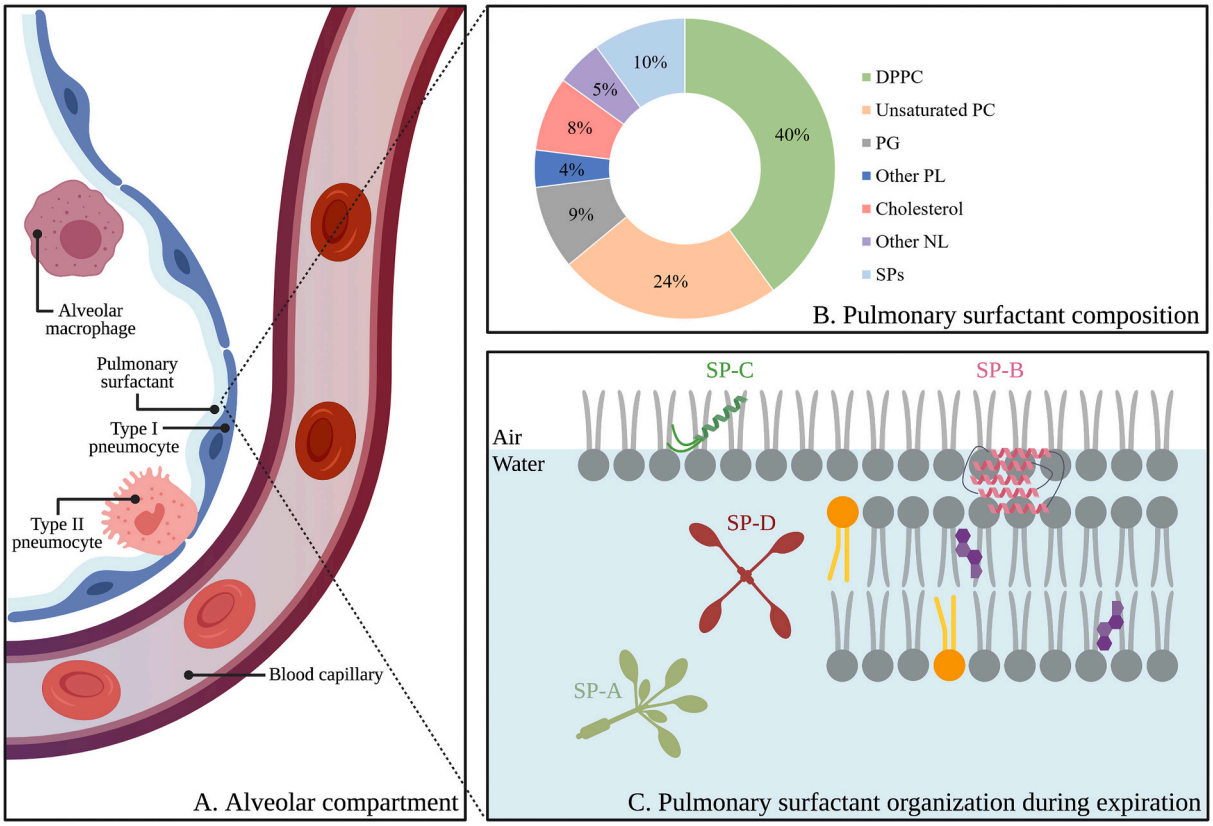

Fig. 2. Schematic representation of the alveolar compartment, including the alveolar epithelium (*i.e.* type I- and type II pneumocytes), alveolar macrophages and pulmonary surfactant **(A)**. Proteolipid composition of pulmonary surfactant (wt%) **(B)**. Organization of pulmonary surfactant and lipid-protein interactions during expiration, according to the squeeze-out model. Grey, orange and purple lipids represent saturated lipids, unsaturated lipids and cholesterol, respectively **(C)**. Abbreviations: DPPC; dipalmitoylphosphatidylcholine, PC; phosphatidylcholine, PG; phosphatidylglycerol, PL; phospholipid, NL; neutral lipid, SPs; surfactant proteins, SP-A; surfactant protein-A, SP-B; surfactant protein-B, SP-C; surfactant protein-C, SP-D; surfactant protein-D. Created with BioRender.com (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vesicles, phospholipids are stored in multivesicular bodies (MVBs) and are eventually directed towards LBs, by which the above-mentioned secretion, reorganization- and adsorption processes resume [70].

3.1.1. *Hydrophilic surfactant proteins*

Next to the well-known biophysical function essential for mammalian breathing, PS also contributes to the pulmonary innate immune system, protecting the alveoli from invading pathogens and other foreign particulate matter [29,65]. These properties are mainly assigned to SP-A and SP-D, two oligomeric proteins that belong to the family of collectins of which the role in the innate immune system can be explained by a dual mode-of-action [65,73]. First, it is reported that SP-A and SP-D have immunomodulatory properties. More specifically, as shown in Fig. 3, these proteins can bind to a variety of receptors on innate immune cells (*e.g.* AMs and monocytes) *via* their carbohydrate recognition domain (CRD) or N-terminal region, thereby modulating cytokine production [74–76]. In addition, SP-A and SP-D can directly bind to soluble pro-inflammatory mediators such as tumor necrosis factor (TNF)α and lipopolysaccharide (LPS), which results in the inhibition of their downstream inflammatory actions (Fig. 3) [65,77,78]. Secondly, SP-A and SP-D bind to carbohydrates, phospholipids and glycolipids present on bacteria, viruses, yeasts and fungi, as well as to neutrophils and DNA *via* their CRD. This so-called 'opsonization' of pathogens and particles results in phagocytosis by immune cells such as AMs (Fig. 3) [79–81]. Next to its crucial role in the protection of alveoli against potentially harmful matter, it is reported that SP-D contributes to surfactant homeostasis, as its absence results in the accumulation of PS in the airways due to an aberrant recycling process, although the exact underlying mechanism is still unknown [82,83].

3.1.2. *Hydrophobic surfactant proteins*

SP-B and SP-C are cationic and amphiphilic proteins, found in and between PS-associated lipid bilayers, and are essential for surfactant adsorption and stabilization at the alveolar air-liquid interface. Both proteins modulate membranes through combined electrostatic and hydrophobic protein-lipid interactions *via* cationic and aromatic residues, respectively [2,69,84,85]. SP-B belongs to the saposin-like protein (SAPLIP) family. All members of this family are characterized by a so-called 'saposin fold', consisting of α-helical domains, six conserved cysteines that form three intramolecular disulfide bridges as well as conserved hydrophobic regions [86]. As depicted in Fig. 2B, the SP-B monomer (79aa) has a complex three-dimensional structure consisting of five cationic amphiphilic α helices that mediate its peripheral orientation in surfactant membranes. Moreover, *via* an intermolecular disulfide bridge, SP-B assembles into a covalent homodimer, which is believed to be important for its surface activity and the promotion of membrane-membrane interactions, of which the latter may induce membrane fusion [87,88].

In addition, SP-B homodimers can assemble into larger oligomers with a ring-shaped channel configuration. These oligomers are believed to mediate translocation of lipids between membranes and towards the air-liquid interface, a key initial step to obtain proper surfactant structure and alveolar dynamics. Moreover, next to its role in dense packing of surfactant components in LBs, it is described that extracellular SP-B also induces the secretion of PS from these intracellular organelles *via* interaction with and activation of receptors on the plasma membrane of

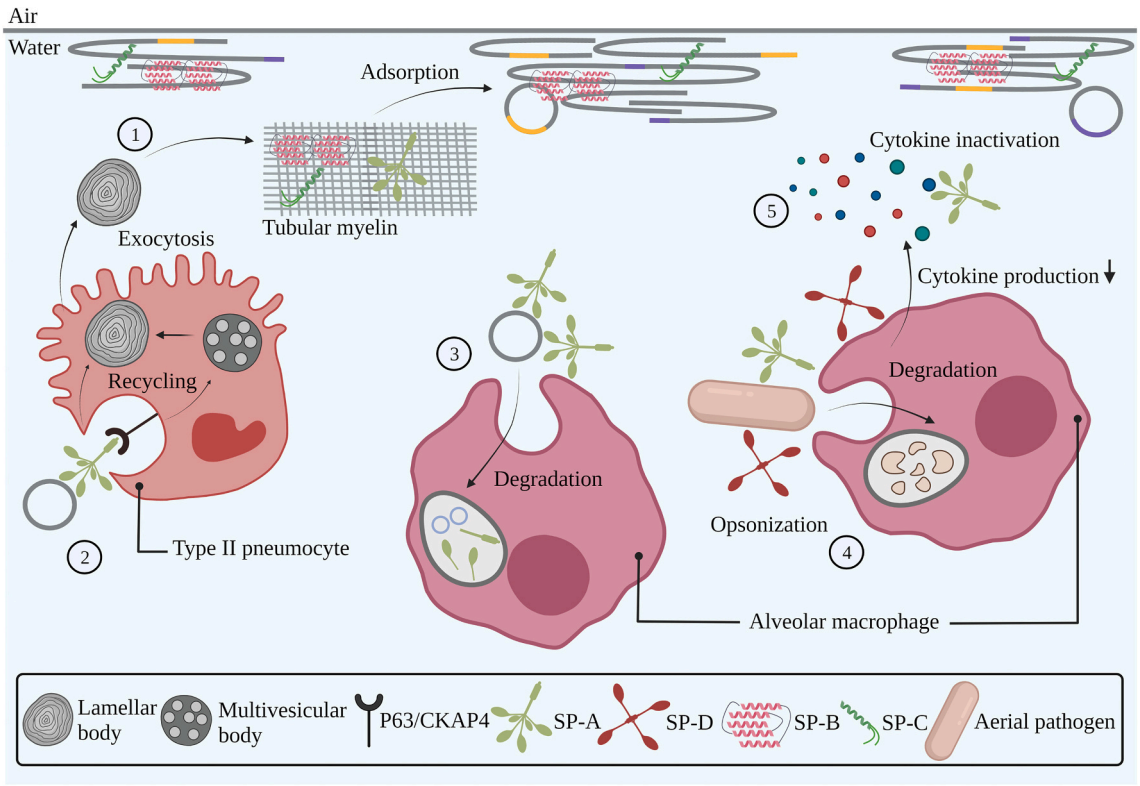

Fig. 3. Interaction of endogenous pulmonary surfactant with pulmonary cells, cellular membranes and the immune system. Pulmonary surfactant is produced by type II pneumocytes in concentrically organized lamellar bodies, which are partly converted into tubular myelin upon secretion into the alveolar lumen. The presence of SP-B and SP-C in tubular myelin drives the adsorption of surfactant membranes towards the air-liquid interface **(1)**. Binding of SP-A to the P63/CKAP4 receptor expressed by type II pneumocytes induces the uptake and recycling of used surfactant components **(2)**. Degradation of used surfactant components occurs *via* uptake and phagocytosis by alveolar macrophages **(3)**. SP-A and SP-D are involved in the pulmonary innate immune system *via* opsonization of aerial pathogens, followed by phagocytosis by alveolar macrophages **(4)**. SP-A and SP-D modulate inflammatory responses *via* interactions with immune cells, thereby reducing cytokine production, as well as *via* direct binding and inactivation of soluble cytokines **(5)**. Grey, orange and purple lines represent saturated, unsaturated and cholesterol-rich domains, respectively. Abbreviations: SP-A; surfactant protein-A, SP-B; surfactant protein-B, SP-C; surfactant protein-C, SP-D; surfactant protein-D. Created with BioRender.com (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

type II pneumocytes. The pleiotropic effects of SP-B at the alveolar air-liquid interface appear indispensable for mammalian breathing, as SP-B deficiency, for example due to mutations in the SP-B gene *SFTPB*, leads to fatal respiratory failure [89,90].

SP-C is a 35aa lipoprotein that, in contrast to SP-B, displays a transmembrane orientation due to its α-helical conformation and the presence of two palmitoylated cysteine residues (Fig. 2B) [91]. Although the absence of SP-C in PS is correlated to chronic respiratory diseases, it does not fully impair breathing as is the case with SP-B deficiency [92,93]. In this regard, although the specific role of SP-C in surfactant layers is not yet fully understood, it is clear that its presence is crucial for proper surfactant structure, recycling and dynamics during repetitive breathing cycles [94,95].

Next to the importance of SP-B and SP-C in surfactant dynamics and stability as described above, recent studies have elucidated the contribution of these proteins, together with phospholipids, to immunomodulatory processes, based on their anti-inflammatory and antibacterial properties [65,96–102]. More in-depth information on the structure and function of the SPs can be found in comprehensive reviews on the topic [83,88,103–105].

3.2. *Exogenous pulmonary surfactant: Surfactant replacement therapy*

PS has become the standard-of-care in the prevention and treatment of NRDS after Avery and Mead discovered the correlation between NRDS and surfactant deficiency [28,106]. Since the lungs of preterm infants are not yet fully developed, there is no production of endogenous surfactant, requiring the supplementation of exogenous clinical surfactants to avoid alveolar collapse and respiratory failure [24,68].

3.2.1. *First-generation synthetic surfactant preparations*

The clinical use of exogenous surfactant is not a recent finding. The potential of SRT was already discovered in 1953, where the nebulization of the non-ionic detergent Triton WR-1339 (Alevaire®) was probed as a treatment for neonatal asphyxia. Since its administration was associated with fatal outcomes, additional efforts were done to improve safety and efficacy of SRT. Further research led to the development of first-generation protein-free synthetic surfactants such as Colfosceril Palmitate (Exosurf®), which contains a mixture of DPPC and two spreading agents (*i.e.* hexadecanol and tyloxapol) [107] and Pumactant (ALEC®), composed of DPPC and PG (Table 1) [108].

3.2.2. *Animal-derived surfactant preparations*

The discovery of the importance of SP-B and SP-C for rapid adsorption and stability of surfactant membranes paved the way towards the use of animal-derived surfactants [113–116]. Indeed, studies that compared animal-derived surfactants and protein-free synthetic surfactants showed that the latter failed to reduce the surface tension, due to the lack of SP-B and SP-C. In addition, it is reported that the administration of animal-derived surfactants results in lower needs for ventilation, reduced risk of pneumothorax and a lower mortality rate. Consequently, protein-free synthetic preparations were withdrawn from the market [117,118].

A variety of animal-derived surfactants, which differ in origin and

Table 1
Protein- and phospholipid composition of surfactant preparations of different origin.

Preparation	Origin	SP-B	SP-C	Phospholipids (PL)	Others	Ref.
First-generation synthetic surfactant preparations						
Colfosceril (Exosurf®)	Synthetic	–	–	13.5 mg/mL (DPPC)	Hexadecanol Tyloxapol	[109]
Pumactant (ALEC®)	Synthetic	–	–	40 mg/mL (DPPC, PG)	–	[109]
Animal-derived surfactant preparations						
Poractant Alfa (Curosurf®)	Minced porcine lung extract	2–3.7 mg/mM PL	5–11.6 mg/mM PL	80 mg/mL	Tripalmitoylglycerol Palmitic acid	[109,110]
Beractant (Survanta®)	Minced bovine lung extract	0–1.3 mg/mM PL	1–20 mg/mM PL	25–30 mg/mL	–	[109,110]
Calfactant (Infasurf®)	Calf lung lavage	5.4 mg/mM PL	8.1 mg/mM PL	35 mg/mL	–	[109,110]
SF-RI I (Alveofact®)	Bovine lung lavage	2–5.6 mg/mM PL	1–12 mg/mM PL	40 mg/mL	–	[109]
Second-generation synthetic surfactant preparations						
Lucinactant (Surfaxin®)	Synthetic	19.8 mg/mM PL (KL4)	–	30 mg/mL (DPPC, POPG)	Palmitic acid	[109]
CHF5633 (Under clinical investigation, phase II)	Synthetic	0.2% (Mini-Bleu)	1.5% (SP-C33Leu)	80 mg/mL (DPPC, POPG)	–	[111,112]
Minisurf (Under preclinical investigation)	Synthetic	3% (SMB)	–	DPPC, POPC, POPG	–	[111]

proteolipid content, are widely used in NRDS management (Table 1). In 1983, Curstedt and Robertson were the first to investigate the porcine-derived Poractant Alfa (Curosurf®) [2,119]. Following studies led to the development of other animal-derived surfactants such as Beractant (Survanta®), Calfactant (Infasurf®) and SF-RI I (Alveofact®), all from bovine origin [103,108,120].

Various studies have compared the efficacy among different animal-derived preparations, however with conflicting results. In general, there are no significant differences between preparations from bovine origin. On the contrary, different studies reported on the superiority of Curosurf®, compared to both Survanta® and Infasurf®, albeit the administered dose of Curosurf® was systematically higher. Therefore, in order to draw clear conclusions, randomized and controlled trials that compare the efficacy of animal-derived surfactants at the same dose are needed [121,122].

3.2.3. *Second-generation synthetic surfactant preparations*

Animal-derived preparations are associated with batch-to-batch variabilities, limited supply and a theoretical risk of pathogen transmission. Together with the fact that extraction and purification from animal lungs are time-consuming and expensive, the focus of SRT has shifted to the development of synthetic alternatives [103]. Due to the complex three-dimensional structure and the high hydrophobicity of SP-B and SP-C, production of their complete native form *via* chemical synthesis or recombinant technologies without loss of function is not yet possible [103,123]. Therefore, there is a need to develop simpler SP-B and SP-C analogues or mimics that can, at least partially, replicate their structure and, most importantly, their biophysical function.

KL4 (21aa, sinapultide) is a cationic amphiphilic peptide with four lysine (K)-tetraleucine (L4) repeats that was clinically approved by the FDA in 2012 as the first synthetic peptide for substitution of SP-B in clinical surfactants. The sequence of KL4 is based on the hydrophobic/hydrophilic ratio in the C-terminal part of SP-B. Upon combination with DPPC, palmitoyloleoylphosphatidylglycerol (POPG) and palmitic acid, this synthetic formulation (Surfaxin®) is as effective as its animal-derived counterparts in the treatment of NRDS [123,124].

Given the importance of both the N- and C-terminal segment for the surface activity of SP-B, Walther and Waring studied the *in vitro* and *in vivo* surface activity of Mini-B (MB) and Super Mini-B (SMB), peptides that comprise two amphiphilic α helices of native SP-B, located at the N- and C termini, which are linked by two disulfide bridges [125]. The SMB peptide additionally contains the seven first N-terminal amino acids (FPIPLPY) [126]. The high hydrophobicity of this sequence fosters interaction with surfactant membranes, explaining the higher surface activity of SMB compared to MB [127,128]. Additional efforts to develop a peptide with increased hydrophobicity and α-helical structure as well as reduced susceptibility to oxidation led to the design of Mini-BLeu, a MB analogue in which two methionine residues are replaced by leucine [129].

In contrast to SP-B, the specific amino acid composition of SP-C seems to be less important for its biophysical function, since SP-C analogues that solely mimic the α-helical structure of the transmembrane part of native SP-C can fully replicate its surface-active properties [130,131]. This observation led to the development of SP-C33Leu, which deviates from native SP-C with respect to the removal or substitution of specific amino acids [103,130,132].

Currently, CHF5633 is the most advanced synthetic surfactant preparation and is under clinical investigation in a phase II trial for NRDS after proof of equal efficacy compared to Curosurf® and Survanta® in *in vitro* and *in vivo* models [133–136]. CHF5633 contains peptide analogues of both SP-B and SP-C (*i.e.* Mini-BLeu and recombinantly synthesized SP-C33Leu), together with DPPC and POPG [2,103]. In addition, a surfactant preparation that contains a mixture of DPPC, palmitoyloleoylphosphatidylcholine (POPC), POPG and SMB (*i.e.* Minisurf), is currently under preclinical investigation [137].

4. Pulmonary surfactant in the fight against COVID-19

4.1. *Surfactant replacement therapy to treat COVID-19-related acute respiratory distress syndrome*

The clinical success of SRT in NRDS has rationalized attempts to broaden the therapeutic application of clinical surfactants to other lung diseases like ARDS. ARDS is a severe form of acute lung injury [25] and is incited by direct lung insults (*e.g.* due to pneumonia or gastric content aspiration) as well as indirect systemic causes (*e.g.* extrapulmonary sepsis, nonthoracic trauma or burn injury) and is associated with high morbidity and mortality [25,138,139]. Clinical manifestations include

surfactant deficiency and inactivation [2,66], pulmonary edema, hypoxemia and suppressed respiration [64]. These symptoms are the result of acute outbursts of pro-inflammatory cytokines and an affected endothelial barrier, which lead to a continuous cycle of pulmonary inflammation and tissue damage [25,39,140–142]. Due to conflicting clinical outcomes, with several clinical trials on adult patients showing no improvement in survival despite enhanced oxygenation, currently no comparable SRT is available to treat patients suffering from ARDS [24,29,30]. The main reason for these suboptimal outcomes is that next to the underlying surfactant deficiency, also inactivation of endogenous and exogenous surfactant occurs due to the presence of surface-active serum proteins and inflammatory molecules in the alveolar lumen, as will be explained later [139].

The high expression level of the ACE-2 receptor on the cellular surface of type II pneumocytes makes this cell type particularly vulnerable to infection with SARS-CoV-2. As a result, SARS-CoV-2 infection is reported to result in decreased endogenous PS levels, as well as altered PS composition and mutations [143]. Of importance, COVID-19-related mortality is mostly caused by a continuous, uncontrolled and exaggerated immune response. This is explained by the recruitment of inflammatory cells such as monocytes and macrophages, which subsequently secrete cytokines and chemokines, resulting in a so-called 'cytokine storm'. Subsequent neutrophil influx and degranulation leads to the destruction of type II pneumocytes and endothelial cells, resulting in the impairment of surfactant production and secretion, a disrupted endothelial barrier, serum leakage into the alveolar spaces and the development of CARDS (Fig. 4) [24–27,144]. Moreover, the ACE-2 receptor is involved in anti-inflammatory and antifibrotic mechanisms, which protect the lung from injuries. Extensive binding of SARS-CoV-2 to this receptor thus reduces its lung-protective capacities [27].

Surprisingly, CARDS does not follow the common clinical pattern in terms of lung mechanics, alveolar damage and radiological presentations typically seen with ARDS [145], but is rather classified as an atypical pneumonia that resembles the pathophysiology of NRDS [24].

Based on the distinctive clinical manifestations of CARDS and the correlation to surfactant deficiency and inactivation, it is clear that results from past ARDS trials cannot simply serve as a reference to its potential in treating patients with CARDS [145]. Therefore, surfactant treatment to anticipate on the progression of severe lung injury in COVID-19 patients has been proposed as a potential treatment for COVID-19 [2,24,30]. Indeed, a study by Gerosa et al. showed that infection with SARS-CoV-2 results in the overexpression of SP-A inside the alveolar spaces. Here, it is important to note that SP-A is present in rather condensed masses, which implies its inactivation or dysregulation [146]. Another study by Islam et al. subsequently showed that, next to affected levels and structures of SP-A, there is a dysregulation of a variety of genes involved in pulmonary surfactant production and activation, as well as its turnover and metabolism [147]. Next to this, Kerget et al. reported a significant increase of SP-D levels in blood samples of infected *vs.* non-infected individuals. Importantly, SP-D levels were higher in patients who developed CARDS and who did not survive SARS-CoV-2 infection. These results highlight the importance of serum SP-D as

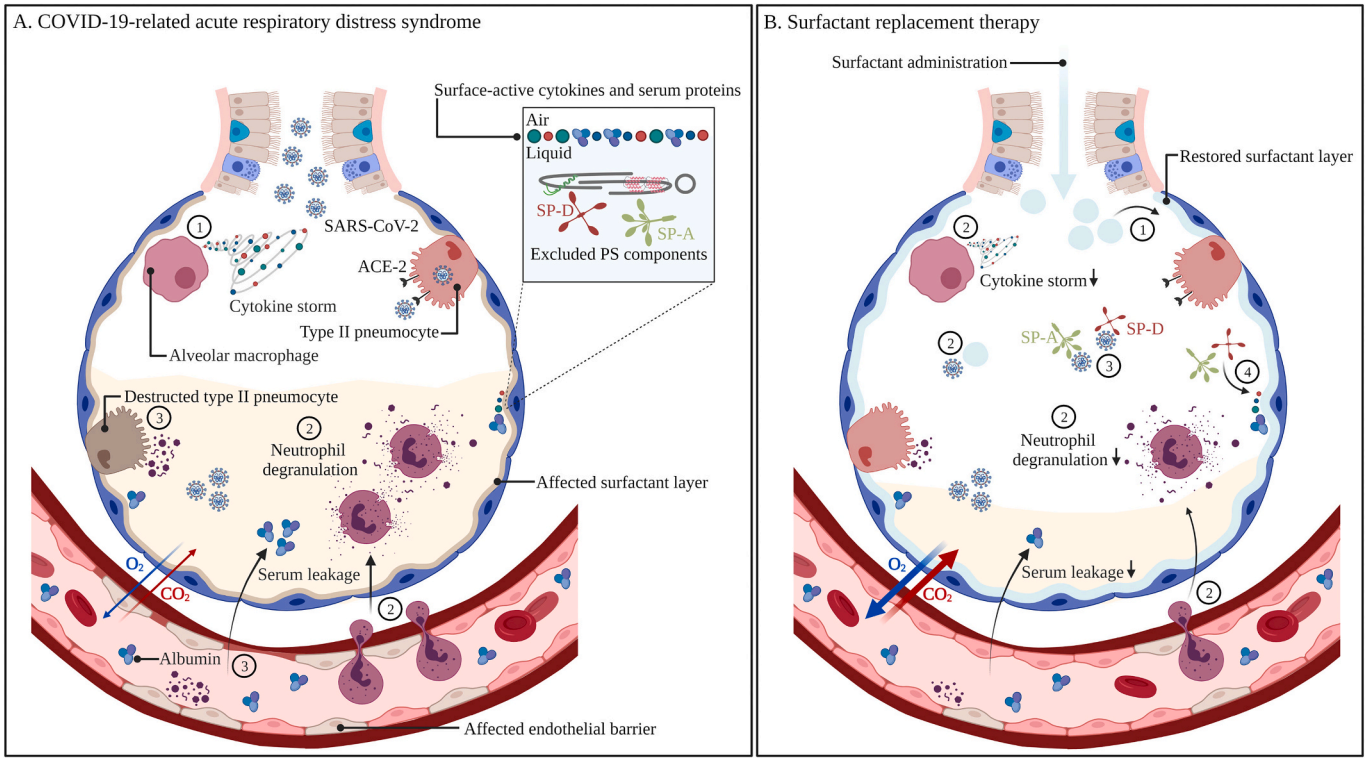

Fig. 4. Non-treated **(A)***versus* pulmonary surfactant-treated COVID-19-related acute respiratory distress syndrome **(B)**. Infection with SARS-CoV-2 results in the recruitment of alveolar macrophages, which produce high levels of cytokines, also referred to as a cytokine storm **(1)**. Subsequent neutrophil recruitment and degranulation **(2)** leads to the destruction of type II pneumocytes and endothelial cells, resulting in reduced surfactant production and secretion, serum leakage in the alveolar spaces and surfactant inactivation by surface-active cytokines and serum proteins that adsorb to the air-liquid interface, thereby excluding endogenous PS components **(3)**. Improper surfactant function leads to collapsed alveoli, aberrant gas exchange and respiratory failure. The administration of exogenous surfactant can supplement the affected endogenous PS pool **(1)**, as well as dampen the inflammatory response *via* interactions with immune cells, cytokines and SARS-CoV-2 **(2)**. Exogenous SP-A and SP-D can prevent viral infection *via* binding and neutralization of the spike protein, thereby preventing its interaction with the ACE-2 receptor on type II pneumocytes **(3)**. Exogenous SP-A and SP-D grants more resistance towards surfactant inactivation **(4)**. Recovery of the surfactant layer as well as reduced inflammation leads to less cellular damage, reduced serum leakage in the alveolar spaces, enhanced gas exchange and thus the prevention of respiratory failure. Abbreviations: COVID-19; coronavirus disease-19, SARS-CoV-2; severe acute respiratory syndrome coronavirus-2, ACE-2; angiotensin-converting enzyme-2, SP-A; surfactant protein-A, SP-D; surfactant protein-D. Created with BioRender.com

a biomarker for disease progression, as well as underscore the potential of SRT in severely ill COVID-19 patients due to the leakage of SP-D from the alveolar spaces into the systemic circulation, induced by an affected alveolar integrity [148]. Likewise, a study by Tong et al. revealed that serum SP-D levels were significantly higher in patients with severe COVID-19 [149].

As shown in Fig. 4, the administration of exogenous surfactants is reported to have several beneficial features, *i.e.* (1) restoration of the endogenous surfactant pool, thereby reducing the surface tension and work of breathing, and (2) mitigating the activation of the innate immune system and preventing inflammatory damage, which is mainly assigned to the presence of anionic lipids in the surfactant preparation. POPG and PI are reported to have immunomodulatory properties *via* interaction with toll-like receptors (TLRs) and viruses. More specifically, *in vitro* experiments revealed that these anionic lipids are capable of suppressing the activation of both TLR2 and TLR4 in AMs [99,150]. In addition, *in vitro* experiments in primary human bronchial epithelial cells (HBECs) showed that both POPG and PI could bind to respiratory syncytial virus (RSV), thereby preventing infection and production of interleukin (IL)-6 and IL-8. These findings were confirmed *in vivo* in RSV-infected mice, where intranasal administration of POPG and PI resulted in a decreased neutrophil- and lymphocyte count in lung lavage, as well as a significant reduction in viral load [98,151,152]. Likewise, *in vitro* studies in HBECs and Madin-Darby Canine Kidney (MDCK) cells showed that POPG and PI prevent infection with H3N2 influenza A virus (IAV). Subsequent *in vivo* studies also demonstrated protection of mice from H1N1-IAV infection [153].

It is worth mentioning that the optimal composition and origin of surfactant preparations for the treatment of CARDS and NRDS might differ. First, all clinical surfactants currently on the market to prevent NRDS are devoid of the hydrophilic proteins SP-A and SP-D, albeit that their presence can add to a more effective treatment of CARDS. SP-A and SP-D could promote the clearance of SARS-CoV-2 through binding of their CRD to the glycosylated spike protein [154], as well as modulate the inflammatory response correlated to infection and associated with disease severity (Fig. 4) [154,155]. Earlier *in vitro* studies already demonstrated that SP-A and SP-D have antiviral properties against CoVs, where SP-A and SP-D were able to bind and neutralize HCoV-229E [156,157]. Furthermore, the antiviral properties of SP-A and SP-D have been confirmed for a variety of other viruses [158–162].

As shown in Fig. 4, the addition of SP-A and SP-D can also grant more resistance to surfactant inactivation [2,163]. A damaged endothelial barrier results in the leakage of serum into the alveolar lumen, as extensively described in severely ill COVID-19 patients suffering from CARDS. In the same manner as surface-active inflammatory molecules (*e.g.* IL-1 and TNF), surface-active serum proteins (*e.g.* albumin) can adsorb to the air-liquid interface, creating a steric and electrostatic barrier that prevents effective surfactant adsorption [164–166]. Interestingly, recent studies have shown that the addition of non-adsorbing hydrophilic macromolecules such as polyethyleneglycol (PEG) and hyaluronic acid also prevents surfactant inactivation in a similar way [139].

Since animal-derived SP-A and SP-D could also induce immunogenic reactions in adults, addition of these proteins to the formulation would require synthesis of human homologues [2]. Efforts to develop recombinant fragments of SP-A (rfhSP-A) and SP-D (rfhSP-D) have been undertaken. Most importantly, these fragments are able to mimic the antipathogenic and immunomodulatory properties of the native proteins [105]. In this regard, a recent study by Madan et al. reported that rfhSP-D successfully neutralized SARS-CoV-2 and reduced infection in clinical samples, where it is more potent in inhibiting viral replication and infectivity compared to remdesivir [167]. In addition, it has been shown that rfhSP-D binds to the S1 subunit of the viral spike protein, thereby preventing its interaction with the ACE-2 receptor [168].

Secondly, focusing on the development of synthetic surfactants might become even more important in the treatment of CARDS. Indeed, synthetic surfactants seem more resistant to inactivation [169]. In addition, it has been reported that CHF5633 reduces LPS-induced pro-inflammatory cytokine release from human monocytes, a valuable property given the marked inflammatory nature of COVID-19 [170–172].

In addition to higher doses and/or more frequent administrations [173], also an alternative pulmonary delivery mode might be required. In neonates suffering from NRDS, surfactants are typically administered *via* an intratracheal bolus instillation, yet this delivery mode is invasive and associated with a non-homogenous distribution throughout the lung tissue. Taking into account the larger surface area of the conducting airways in adults, there is a risk for more significant loss of surfactant along its way to the alveoli. Therefore, non-invasive delivery modes such as surfactant aerosolization using ultrasonic or jet nebulizers are currently under development [174–177], and have already proven to be safe in animal models without functional alterations of the surfactant preparation [178–180].

Based on the above-mentioned beneficial features of surfactant administration to COVID-19 patients, several clinical trials have been initiated to investigate the safety, efficacy and practical feasibility of this treatment option and are summarized in Table 2 [2,181].

In addition, the off-label use of surfactants to treat CARDS has already been reported in the literature.

Busani et al. reported on the administration of Curosurf® to five critically ill patients suffering from COVID-19-related pneumonia and low lung compliance. Patients received surfactant during one month at a dosage of 30 mg/kg *via* intratracheal intubation, initiated shortly after the onset of invasive mechanical ventilation. The results showed improvement of oxygenation after one hour in four patients and after six hours in all patients. Additionally, over the course of 30 days, a survival rate of 80% was observed despite the critical condition of the included patients [181].

A single-center retrospective case-control pilot study was reported by Piva et al., where seven COVID-19 patients suffering from CARDS received Curosurf® through bronchoscopy for two months at a dose of 720 mg in 150 mL normal saline for a total of ten instillations. These patients were matched to 14 COVID-19 patients with similar disease severity, receiving only supportive care. Despite the infection risk for healthcare providers related to the bronchoscopy procedure, the personnel did not develop COVID-19, indicating the feasibility of surfactant therapy *via* this delivery mode. In addition, a favorable safety profile was observed, where none of the patients receiving surfactant developed acute decompensation. Despite the limited and preliminary data regarding treatment efficacy, surfactant delivery and retention in the lungs following bronchoscopy was confirmed. However, future clinical trials using this mode of delivery are required to confirm a possible reduction in total time on mechanical ventilation and long-term mortality [182].

Heching et al. reported on the administration of Infasurf® at a dosage of 20 mg/kg to a critically ill COVID-19 patient. Surfactant was directly dispersed into the lungs *via* a tracheobronchial suction catheter passed through the endotracheal tube. An improvement of oxygenation was seen after 18 h, which further improved after 48 h and resulted in the removal of the patient from extracorporeal membrane oxygenation (ECMO) and extubation [29].

Although validation on the efficacy, safety and feasibility by sufficiently powered clinical trials is required, the outcomes as mentioned above already give an optimistic indication on the potential of exogenous surfactant administration to COVID-19 patients.

4.2. Use of exogenous pulmonary surfactant for vehiculization and spreading of COVID-19-targeting drugs

As the lungs are the major target tissue of SARS-CoV-2 infection, pulmonary delivery of COVID-19-targeting drugs is an attractive alternative for intravenous administration. Local application to the lung has

Table 2
Overview of ongoing clinical trials investigating the potential of surfactant replacement therapy in COVID-19 management. Abbreviations: NA; not announced, BLES; bovine lipid extraction surfactant.

Preparation	Dose	Dose frequency	Initiation	Delivery mode	Sample size	Phase	Ref.
Bovactant (Alveofact®)	1080–3240 mg/kg (45 mg/mL)	3/day	Within 24 h of ventilation	Nebulization	24	NA	[31]
BLES®	50 mg/kg (27 mg/mL)	≤ 3/day	As soon as possible/within 48 h of ventilation	Intratracheal instillation	20	I/II	[32]
Poractant Alfa (Curosurf®)	48 mg/kg (16 mg/mL)	NA	Within 72 h of ventilation	Endobronchial administration	20	II	[33]
Lucinactant (Surfaxin®)	80 mg/kg	NA	At the time of ventilation	Intratracheal instillation	30	II	[34]
Poractant Alfa (Curosurf®)	30 mg/kg (80 mg/mL)	3/day	Within 48 h of ventilation	Intratracheal instillation	85	II	[35]

a variety of advantages as it is non-invasive, grants direct access to target cells, allows a rapid onset of action and prevents systemic side effects [183,184]. A variety of new and existing drug molecules and approaches are envisioned as a potential treatment for COVID-19, including antiviral therapies as well as therapeutics that target pathophysiological pathways associated to infection [2]. In this regard, this section will discuss the potential to exploit exogenous PS as a drug delivery vehicle or carrier to enhance drug spreading and adsorption or drug targeting to specific cell types after local pulmonary delivery, leading to improved therapeutic outcomes.

4.2.1. Antivirals

Antiviral drugs might be most suitable for treating COVID-19, as they specifically target the viral life cycle and viral spreading. As the pandemic unfolded, the number of clinical trials that evaluated the repurposing of existing drugs as antivirals concurrently increased, since this repurposing strategy has several advantages compared to the development of new drugs [7,185].

However, the randomized evaluation of COVID-19 therapy (RECOVERY) trial showed no clinical benefit from the use of hydroxychloroquine or lopinavir/ritonavir [186,187]. As a result, Gilead's remdesivir, a repurposed antiviral drug that acts as a nucleoside analogue to inhibit the RNA-dependent RNA polymerase of SARS-CoV-2 [188], is the only antiviral drug approved for the treatment of COVID-19 thus far. The approval of remdesivir was grounded on three randomized controlled trials, where the administration of remdesivir resulted in a shorter recovery time and a reduction in respiratory tract infection compared to the placebo group.

4.2.2. Antibodies

Another approach to prevent viral entry is the development of mAbs or antibody-based products that for example bind the viral spike protein, thereby blocking its interaction with the ACE-2 receptor. In this regard, Wrapp et al. reported successful neutralization of the SARS-CoV-2 spike protein by heavy chain-only antibodies (HCAbs). Moreover, by the construction of a bivalent Fc-fusion (*i.e.* a tail-to-head combination of two HCAbs fused to the Fc domain of human IgG1), it also neutralizes SARS-CoV-2 pseudoviruses [52]. Likewise, Koenig et al. screened an HCAb library, where four HCAbs seemed to efficiently neutralize SARS-CoV-2 *via* binding to two distinct epitopes on the RBD, which allows combination of HCAbs to obtain improved neutralization efficiencies. In this regard, a bivalent HCAb (*i.e.* VHH VE) was able to neutralize SARS-CoV-2 by a rather unusual mode-of-action. Here, the binding of VHH VE to the RBD induced fusion, leading to irreversible changes in spike protein structure, thereby preventing its subsequent interaction with the ACE-2 receptor on host cell membranes. Importantly, VHH VE was also able to neutralize SARS-CoV-2 variants [189]. Focusing on inhalation therapy, HCAbs have several advantages compared to conventional mAbs. First, they have a high robustness as they are able to refold after denaturation. In addition, they are small and have a high thermal- and chemical stability and solubility. Next to targeting the spike protein, also neutralization of inflammatory molecules such as interleukins can be of interest. In this regard, the RECOVERY trial probed the potential of tocilizumab, a recombinant humanized anti-IL-6 mAb. Results showed improved survival in hospitalized COVID-19 patients with confirmed hypoxemia and systemic inflammation [190].

4.2.3. Corticosteroids

Since COVID-19 disease severity is correlated to inflammatory lung injury, the administration of corticosteroids is an arguable treatment option to temper inflammation pathways and prevent the development of respiratory failure. In this regard, the RECOVERY trial investigated the impact of oral and intravenous administered dexamethasone (once daily, 10 days) on the 28-day mortality. Dexamethasone treatment resulted in a lower mortality rate in patients that required invasive mechanical ventilation or oxygenation, compared to patients receiving usual care. As a result, next to remdesivir, also dexamethasone was approved for hospitalized COVID-19 patients requiring oxygenation with or without mechanical ventilation. Given the many advantages of local pulmonary delivery as described above and its wide application in other respiratory diseases including asthma and COPD, also inhalation corticosteroids (ICS) have been considered for local COVID-19 treatment. Ramakrishnan et al. performed a randomized open-label controlled phase II trial (*i.e.* steroids in COVID-19, STOIC) of inhaled budesonide within the first week of mild COVID-19 symptom onset. Results showed that early administration of inhaled budesonide reduced the hazard of urgent medical care requirements and shortened the recovery time, compared to patients receiving conventional care [191]. A recent study by Yamaya et al. showed that pre-treatment of primary human nasal- and tracheal epithelial cells with a combination of budesonide, glycopyrronium and formoterol lowered viral titers of HCoV-229E and modulated infection-induced inflammation by decreasing the release of IL-6, IL-8 and IFNβ [192]. In addition, budesonide and formoterol lowered TNFα release induced by other respiratory viruses such as rhinoviruses and RSV in bronchial epithelial cells *in vitro* [193]. Notably, recent studies reported on the clinical potential of inhaled ciclesonide. Upon local administration, this corticosteroid has an anti-inflammatory effect as well as antiviral activities, as it lowers SARS-CoV-2 replication and cytotoxic potential in Vero cells *in vitro* [194]. Following this data, Covis Pharma group recently announced favorable safety and efficacy results from a double-blind randomized controlled phase III trial investigating ciclesonide administration *via* a metered-dose inhaler (MDI) in non-hospitalized, symptomatic COVID-19 patients. Results showed faster relief of symptoms and a reduced risk for hospitalization by day 30, compared to placebo. Importantly, no adverse events were reported up to 60 days post-treatment [195]. To improve therapeutic results upon inhalation, Lammers et al. described the promise of dexamethasone nanomedicines. As the encapsulation of drugs into nanoparticles results in enhanced phagocytosis by AMs, dexamethasone could be more specifically delivered to the cells that

play a key role in lung inflammation [196].

4.2.4. Pulmonary surfactant-assisted drug delivery and spreading

In general, despite the availability of many interesting drugs to treat COVID-19, deep penetration of drugs into the lungs and drug deposition in the alveolar spaces is challenging. Impaction of aerosolized drugs on bifurcations will lead to mucociliary clearance and hence subtherapeutic outcomes. Most important, as described above, critically ill COVID-19 patients additionally suffer from acute respiratory distress, which is correlated with the development of hypoxic, collapsed lung regions. These regions are even more difficult to reach following inhalation therapy due to a reduced surface area that is accessible for drug deposition and the inability of the patients to properly use inhalation devices [36].

Therefore, recent studies have evaluated the use of exogenous PS as a vehicle or liposomal carrier for inhalation therapy of COVID-19-targeting drugs. Upon so-called 'surfactant vehiculization' (*i.e.* co-delivery of drugs and an exogenous surfactant preparation, Fig. 5), one can expect improved drug spreading and adsorption along the entire respiratory epithelial surface due to the excellent and rapid surface-spreading of PS, followed by drug release during consecutive compression/expansion cycles upon breathing [2,37]. Owing to the ability of exogenous surfactant to improve lung dynamics as mentioned above, this treatment strategy can be seen as a double-edged sword to attain improved therapeutic outcomes and provides new opportunities for local pulmonary drug delivery, *e.g.* in CARDS.

In addition, liposomal surfactant carriers (*i.e.* drugs formulated inside surfactant liposomes, Fig. 5) can mediate active targeting of drugs. As mentioned above, during consecutive breathing cycles, used PS components are released from surfactant films and recycled through uptake into type II pneumocytes and repackaging in LBs. Next to this, used PS components can also be processed and degraded by AMs. In case the PS-encapsulated drugs are carried along with this depleted pool of PS components, they can also benefit from this recycling mechanism to promote further spreading along the lung surface or enable internalization by these specific cell types [39,40,197].

Given the amphiphilic nature of PS, a variety of therapeutics with different physicochemical properties can be incorporated, even enabling combination therapy by loading several drugs into an exogenous lung surfactant formulation, as shown in Fig. 5 [198]. Hidalgo et al.

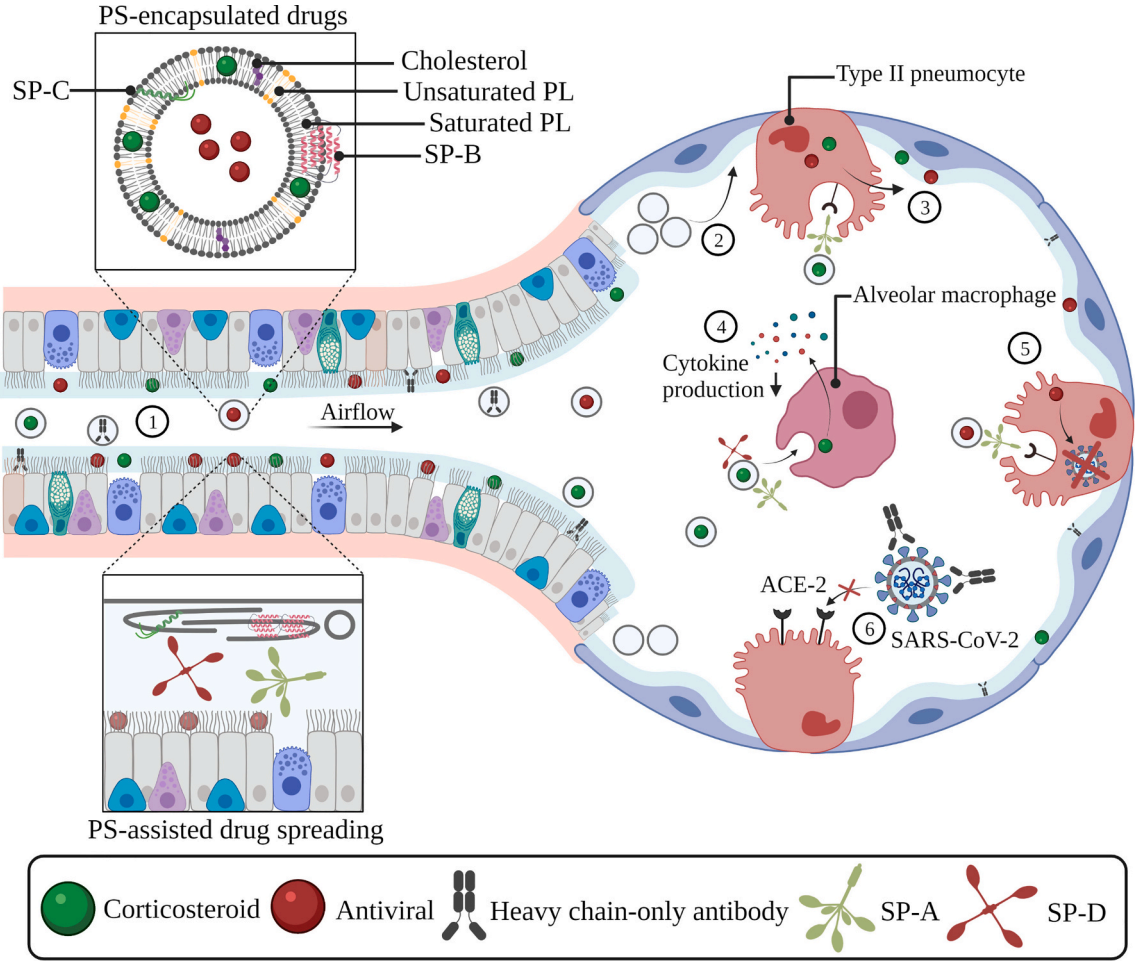

Fig. 5. Exogenous pulmonary surfactant as a vehicle or liposomal carrier for COVID-19-targeting drugs. Encapsulation of drugs inside exogenous PS can improve drug spreading and adsorption along the entire pulmonary epithelium **(1)**. The administration of exogenous surfactant can supplement the reduced or inactivated endogenous surfactant pool, which prevents alveolar collapse and facilitates the delivery and deposition of inhaled drugs in deeper lung regions **(2)**. SP-A-mediated uptake in type II pneumocytes induces drug- and surfactant recycling, which allows further drug spreading along the alveolar interface **(3)**. SP-A- and SP-D-mediated drug internalization by alveolar macrophages allows anti-inflammatory drugs (*e.g.* corticosteroids) to interfere with the production of pro-inflammatory cytokines **(4)**. SP-A-mediated uptake of antivirals in type II pneumocytes results in the reduction of viral replication processes and/or viral infectivity *via* various mechanisms of action **(5)**. PS-assisted delivery of monoclonal-antibody based products (*e.g.* heavy chain-only antibodies) allows them to bind to viral components (*e.g.* the spike protein), thereby preventing interactions with the ACE-2 receptor thus reducing their infectivity **(6)**. Abbreviations: SP-A; surfactant protein-A, SP-B; surfactant protein-B, SP-C; surfactant protein-C, SP-D; surfactant protein-D, ACE-2; angiotensin-converting enzyme-2, SARS-CoV-2. severe acute respiratory syndrome coronavirus-2, PL; phospholipid, PS; pulmonary surfactant. Created with BioRender.com

confirmed that hydrophobic drugs such as corticosteroids experience enhanced transport and spreading along the respiratory surface upon formulation in exogenous PS [199]. In addition, Ya-Min et al. compared the therapeutic effect of aerosolized dexamethasone and co-aerosolization of dexamethasone and PS in an acute lung injury (ALI) mouse model. While dexamethasone alone did not improve lung function in terms of PaO_2, co-delivery with PS resulted in a reduction of lung index, lung injury scores and levels of the pro-inflammatory cytokines TNFα, IL-1β and IL-6 in bronchoalveolar lavage (BAL) [198].

PS-assisted pulmonary drug delivery of other small molecules has also been investigated. Here, the immunomodulating agent tacrolimus retained its immunosuppressive efficacy after lung transplantation and in respiratory infections upon encapsulation in lung surfactant-like carriers. In addition, the combination of the aminoglycoside antibiotic tobramycin and clinical surfactant leads to improved survival rates in mice suffering from *Klebsiella pneumonia* infection [200,201]. Next to small molecules, also the delivery of biological drugs including antimicrobial peptides, antibodies, antioxidant enzymes and viral particles have shown to benefit from this particular encapsulation approach [202–205].

A recent publication reported the potential of co-aerosolizing the cationic amphiphilic compound ambroxol and surfactant in the form of ambroxol-loaded- and coated DPPC nanoparticles as a treatment for CARDS. Ambroxol is a mucolytic drug that is widely used in a variety of pulmonary diseases [206]. Moreover, it is reported to have anti-inflammatory, anti-oxidant, antiviral and antibacterial properties, as well as to stimulate the production- and secretion of PS [207,208]. Upon co-delivery, the surfactant fraction can supplement the deficient or inactivated endogenous surfactant pool, while ambroxol can further fight the disease by the array of therapeutic properties as described above. This approach was established by an earlier meta-analysis on the administration of high-dose ambroxol to patients suffering from ARDS, of which results showed an improvement in oxygenation, as well as a reduction of superoxide dismutase (SOD), TNFα and IL-6 levels in serum [209].

4.3. *Use of exogenous pulmonary surfactant to promote delivery of antiviral siRNA molecules*

4.3.1. *siRNA therapeutics as an effective antiviral therapy*

As briefly mentioned above, there is a clear unmet medical need for more specific anti-SARS-CoV-2 therapies targeting key steps in the viral life cycle. A promising strategy is the use of the RNA interference (RNAi) mechanism, where siRNA molecules can be designed to specifically and selectively target viral and/or host-related proteins, without affecting host cell homeostasis [210].

Upon inhalation and delivery into the target cell cytosol, these double-stranded siRNA molecules will bind to the RNA-induced silencing complex (RISC). After digestion of the lagging strand, the activated RISC employs the retained guide strand to bind to a complementary region on the target mRNA, followed by its enzymatic degradation. Notably, this approach ensures a very effective treatment due to the catalytic nature of this gene silencing mechanism, requiring relatively few siRNA molecules per cell to induce potent gene knockdown [211–215]. In addition, the screening of siRNA libraries to identify lead molecules with promising antiviral effects is not as time-consuming as the *de novo* development of small molecules or antibody(−based) therapeutics, an attractive feature given the rapid spread of viral infections such as SARS-CoV-2 [17].

Efforts to exploit the RNAi mechanism to treat respiratory infections have already been made, with a first report by Bitko and Barik in 2001 showing successful *in vitro* antiviral activity against RSV *via* siRNA-mediated knockdown of the RSV phosphoprotein (*i.e.* the smaller subunit of RNA-dependent RNA polymerase) [216]. In addition, to anticipate on the outbreaks of SARS-CoV-1 and MERS-CoV, a variety of siRNA molecules have been designed that effectively target viral RNA-dependent RNA polymerase, helicase, proteolytic enzymes and structural proteins [217].

As briefly mentioned above, both a variety of SARS-CoV-2 proteins responsible for infectivity or viral replication as well as host-related proteins that play a crucial role in viral infection or disease severity are interesting siRNA targets for an effective antiviral therapy. In severe cases, COVID-19-related morbidity and mortality is caused by an exaggerated pulmonary immune response. More specifically, an upregulation of INFγ, TNFα, IL-6, IL-10 and IL-12 is reported, where especially IL-6 seems to promote the inflammatory cascade [58,218–221]. In addition, the upregulation of high-mobility group box 1 (HMGB1) and sphingosine-1-phosphate lyase (S1PLyase) is observed, where the latter has a more upstream mode-of-action, as its downregulation results in the reduction of TNFα- and IL-6 levels [141,142]. Other studies additionally report on increased levels of IL-2, IL-7, granulocyte-colony stimulating factor (G-CSF), inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), where IP-10 is correlated to disease deterioration and death [51,222,223].

Although downregulation of the inflammatory cascade is a warranted approach to reduce disease severity, translational suppression of viral proteins might be a more direct and specific strategy. The genomic sequence of SARS-CoV-2 was released early after its discovery, which facilitated the development of siRNA libraries to target SARS-CoV-2 viral ssRNA coding regions. Currently, several companies are focusing on the development of siRNA molecules to target SARS-CoV-2. Alnylam Pharmaceuticals has synthetized more than 350 siRNA hits that target highly conserved regions in the SARS-CoV-2 genome [224]. Likewise, OilX Pharmaceuticals holds a patent on 30 siRNA molecules targeting conserved regions among coronaviral transcripts. Lastly, Sirnaomics focuses on the development of siRNA molecules targeting proteins involved in SARS-CoV-2 infection and replication [225].

RNAi-based suppression of all four structural proteins (S, E, M and N) can avoid cellular entry and viral assembly, where targeting the spike- and envelope protein have attracted most attention. Since the spike protein is responsible for viral entry into host cells, downregulating this protein will result in its absence in newly assembled viral particles, leading to exocytosis of viruses with reduced infectivity [225]. In this regard, Gallicano et al. showed successful *in vitro* spike protein suppression in HEK293 cells and human airway tracheal cells, without compromising cell growth and viability [226]. Next to this, the envelope protein is involved in viral assembly and release into the extracellular environment. The sequence of the envelope protein seems to be highly conserved [225], which allows the development of siRNA molecules that will not lose their efficiency upon mutation of the virus, an important consideration taking into account the emergence of SARS-CoV-2 variants [227].

Next to structural proteins, also proteins involved in viral replication are envisioned. In this regard, the sequences coding for protease 3CL, RNA-dependent RNA polymerase and helicase are known to be highly conserved among β-coronaviruses [225].

It is worth noting that the RNAi mechanism allows the combination of different siRNA molecules into one synergistic treatment. In this way, multiple viral- and host-related genes can be targeted simultaneously, leading to significantly enhanced therapeutic efficiencies [228–231].

4.3.2. *Nanoparticle-assisted cytosolic delivery of siRNA molecules following inhalation therapy*

After inhalation, siRNA molecules need to be safely guided to the target cell cytosol, for which they are generally formulated into nanoparticles. However, besides the many advantages of local pulmonary delivery of nanomedicines, the lung also poses many extra- and intracellular barriers that can hamper siRNA delivery [232,233]. In general, predicting the fate of inhaled nanoparticles is complex since it is determined by a variety of parameters. Next to particle-related properties (*e.g.* mean mass aerodynamic diameter (MMAD), hydrophobicity

and charge), also patient-related properties (*e.g.* breathing patterns) as well as the anatomy, physiology and pathological state of the lung are involved. An important anatomical barrier towards pulmonary siRNA delivery is the highly branched structure of the lungs, where the fate of aerosolized particles following inhalation therapy will strongly depend on their MMAD. Nanomedicines formulated in aerosolized microparticles (*e.g.* dry powders or nebulized droplets) that sediment in the bronchial or alveolar region will subsequently need to cross the respiratory mucus or pulmonary surfactant layer to reach the underlying bronchial or alveolar epithelial cells, respectively [234–236]. In addition, nanoparticles are prone to clearance by respiratory immune cells such as macrophages, which can be present at both interfaces [237]. In this regard, investigating nanoparticle/PS interactions are crucial since such interactions can both alter the nanoparticles' distribution profile, targeting potential and drug delivery efficiency, as well as affect the endogenous PS layer [83]. However, the design of such studies is challenging since it requires the use of materials or models that closely resemble the composition of endogenous PS. Indeed, clinically used surfactants can be obtained easily but have several limitations; they are devoid of the hydrophilic proteins SP-A and SP-D and both the phospholipid- and SP-B/SP-C fractions are often lower compared to physiological conditions. Therefore, the use of patient-derived PS (*e.g. via* BAL) is more desired, however requires more invasive procedures. Several studies report on the greater affinity of surfactant proteins to smaller particles, leading to enhanced toxicity of these particles towards endogenous PS [238–240]. In this regard, SP-A and SP-D will more easily adsorb to hydrophilic nanoparticles, leading to particle opsonization and enhanced uptake by AMs and lung dendritic cells [241,242]. In addition, Wohlleben et al. showed that SP-A adsorption may induce interactions with PS lipids, thereby facilitating particle diffusion through the liquid phase and enhancing particle delivery to desired cell types [243]. On the other hand, SP-B and SP-C have a higher affinity for hydrophobic particles, which is more detrimental for the biophysical function of PS given the importance of SP-B and SP-C in surfactant adsorption and stability [197]. In this context, cationic and hydrophilic particles seem more biocompatible since their intrinsic properties mitigate interactions with SP-B and SP-C [244–246]. Importantly, Beck-Broichsitter et al. showed that pre-coating of particles with PS components such as SP-B and SP-C can avoid their interaction with endogenous PS, thus reducing the risk for PS toxicity or a change in the particles' characteristics [246]. In contrast to the adsorption of surfactant proteins, Raesch et al. elucidated that the composition of the lipid corona was independent from the particles' properties [234].

However, one of the most difficult barriers to overcome for siRNA therapeutics is situated at the intracellular level. When nanoparticles interact with the cellular plasma membrane, they are typically taken up *via* endocytosis, confining them inside early endosomes. These organelles further mature towards late endosomes and finally fuse with lysosomes, which are characterized by an acidic pH (~4.5–5) and the presence of degrading enzymes, including nucleases [247]. As the RNAi machinery resides in the cytosol, endosomal escape of nanoparticles and/or their siRNA cargo into the cell cytosol before lysosomal fusion occurs is required to induce target gene silencing. Despite the many efforts towards the design of effective nanoformulations in the last decades, even for state-of-the-art nanocarriers the endosomal escape efficiency remains very low (< 1–2%) [44]. Importantly, since the lungs of COVID-19 patients are already highly sensitized due to the activation of inflammatory pathways, not only an effective, but also a biocompatible nanoparticle for inhalation therapy of siRNA therapeutics is highly sought after. In this regard, exploiting bio-inspired materials is a research area gaining attention.

As mentioned above, PS can be regarded as an extracellular barrier upon inhalation of nanomedicines. More specifically, surfactant components can adsorb to the surface of the nanoparticle, creating a biomolecular corona. In addition, due to the presence of negatively charged surfactant components (*e.g.* PG, PI and PS), there is a risk for nanocarrier aggregation or siRNA decomplexation prior to endocytic uptake [37,83,197,248]. In this regard, De Backer et al. investigated the colloidal stability and transfection efficiency of siRNA-loaded cationic dextran nanogels (siNGs) in the presence of two clinically available animal-derived surfactants (*i.e.* Curosurf® and Infasurf®) [45]. These polysaccharide nanoparticles are biodegradable due to the presence of carbonate ester crosslinks that interconnect the dextran backbone and were previously shown to have a high loading capacity for siRNA molecules [249]. Moreover, siNGs showed efficient delivery of siRNA *in vitro* in lung epithelial and alveolar macrophage cell lines (*i.e.* H1299_eGFP and MH-S) [250].

Despite a significant reduction of siNG cellular uptake upon surfactant exposure, gene silencing was maintained in both cell lines, indicating that the presence of surfactant can further promote the cytosolic delivery of siNGs. These initial results led to the development of a bio-inspired hybrid nanoparticle composed of a siNG core layered with a surfactant shell (Curosurf®). In addition to the above-mentioned beneficial effects of PS (*i.e.* surfactant supplementation, improved biodistribution and anti-inflammatory effects) as well as the promoted siRNA delivery, this anionic surfactant coating is expected to improve *in vivo* stability (*e.g.* mitigating the interaction with and decomplexation of siRNA by mucins and endogenous PS components), as well as nanocarrier biocompatibility [46].

Following *in vivo* studies that probed the administration of uncoated- and Curosurf®-coated siNGs *via* pharyngeal aspiration in mice showed that only the latter could induce gene suppression in resident AMs, reaching 70% knockdown of the pan-leukocyte marker CD45 at a dosage of 1 mg/kg [47].

Clinical translation of the above-mentioned core-shell nanocomposites requires testing the long-term stability as well as the delivery efficiency following aerosolization. In this regard, previous studies already showed the feasibility to lyophilize PS preparations of different origins without compromising their surface-activities [251–253]. Considering this, Merckx et al. assessed lyophilization of Curosurf®-coated siNGs, followed by reconstitution and nebulization using a state-of-the-art vibrating mesh nebulizer. Importantly, neither the lyophilization and reconstitution process, nor the subsequent nebulization negatively impacted the physicochemical properties and the biological performance of the particles. Of note, no stabilizing agents such as cryo- and lyoprotectants were required to achieve this feat [48].

Further research by Merckx et al. revealed that the cationic amphiphilic SP-B is the key component in lung surfactant responsible for the improved siRNA delivery by siNGs, which was demonstrated both *in vitro* and *in vivo* in an LPS-induced acute lung injury model, targeting the pro-inflammatory cytokine TNFα (Fig. 6) [49]. This discovery enabled to reduce the complexity of the surfactant coat by replacing Curosurf® with a simplified SP-B proteolipid mixture. In addition, the latter enabled to investigate the impact of the lipid components on the delivery-promoting activity of SP-B in more detail. *In vitro* studies demonstrated the importance of a fluid lipid bilayer to support SP-B, with cholesterol not exceeding physiological levels, while the type of anionic lipid was less critical [50].

Guagliardo et al. further revealed that SP-B fosters endosomal escape of siRNA *via* membrane fusion events. This mode-of-action in part explains the enhanced *in vitro* gene silencing efficiency when SP-B is present in a more fluid lipid microenvironment, as the latter allows lateral diffusion of SP-B molecules and the formation of SP-B homodimers or larger oligomers with improved membrane-interaction potential [254,255]. More specifically, *in vitro* results showed that SP-B-mediated fusion was pH independent and required the presence of anionic lipids in the opposing membrane. As shown in Fig. 7, these anionic lipids are believed to electrostatically interact with the cationic SP-B, guaranteeing close membrane contact to provoke membrane fusion followed by cytosolic delivery of encapsulated siRNA molecules. Interestingly, a similar mechanism of action is described for arginine-rich cell penetrating peptides (CPPs), as these residues allow electrostatic interactions

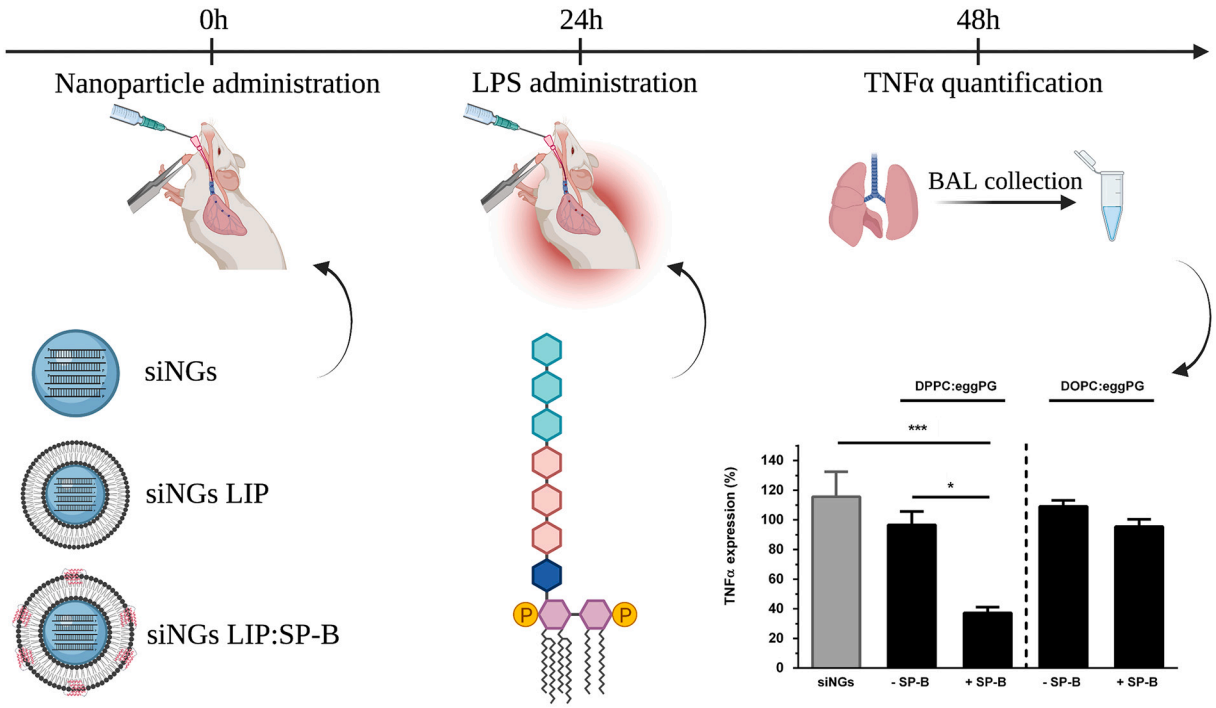

Fig. 6. Schematic overview of relative TNFα silencing in a murine, LPS-induced acute lung injury (ALI) model. Intratracheal administration of anti-TNFα siRNA was performed using uncoated nanogels (siNGs) or nanogels coated with a surfactant-inspired proteolipid composition (DPPC or DOPC:PG 85:15, LIP), with or without SP-B (siNGs LIP, siNGs LIP:SP-B), followed by LPS administration after 24 h. TNFα levels were quantified in BAL fluid, obtained 24 h after LPS stimulation. TNFα expression levels of mice treated with anti-TNFα siRNA are normalized to mice treated with control siRNA (siCTRL). Only siRNA delivery using siNGs coated with DPPC:PG and supplemented with SP-B leads to substantial gene silencing. All values are a mean ± standard deviation (SD) from four independent repeats (n = 4). Statistical analysis was performed via One-Way ANOVA followed by a Tukey's multiple comparison test. Abbreviations: TNFα; tumor necrosis factor α, LPS; lipopolysaccharide, siNGs; siRNA-loaded nanogels, SP-B; surfactant protein-B, BAL; bronchoalveolar lavage. Data adopted from [49], with permission. Created with BioRender.com

with anionic lipids in the endosomal compartment. For example, a disulfide-bonded dimer of HIV-TAT (*i.e.* dHIV-TAT) fosters endosomal escape by binding to bis(monoacylglycerol)phosphate (BMP) lipids in the late endosomal compartment. Upon binding, fusion occurs between bilayers containing BMP, followed by content leakage and endosomal escape of the cargo [256].

Similar to SRT, also this treatment approach might benefit from the development of SP-B analogues that can mimic the siRNA delivery properties of native SP-B. In this regard, Qiu et al. showed succesfull siRNA delivery into alveolar- and bronchial epithelial cells (*i.e.* A549 and BEAS-2B) *in vitro* upon complexation with the cationic KL4 peptide [257]. Following studies by the same group showed *in vitro* mRNA delivery in lung epithelial cells *via* complexation with a (12-mer) PEGylated KL4 peptide. To obtain a suitable microformulation for local pulmonary delivery, these complexes were formulated into a dry powder without compromising the delivery efficiency of the particles. Intratracheal administration of the mRNA/PEG_{12}KL4 complexes *via* a liquid or powder aerosol in mice led to succesful mRNA delivery in deeper lung regions without inducing inflammation [258].

5. Conclusions

Although the COVID-19 pandemic is gradually losing strength due to global vaccination initiatives, the slow-moving global vaccine distribution, residual infections of vaccinated, non-vaccinated and immunosuppressed individuals as well as the possible emergence of resistant viral variants advocate the continued development of effective COVID-19 treatment strategies. In this regard, pulmonary administration of exogenous pulmonary surfactant (*i.e.* SRT) could greatly contribute to COVID-19 management as these preparations could supplement the deficient or inactivated endogenous surfactant pool as well as directly target SARS-CoV-2 and alleviate inflammation-related symptoms upon combination with small molecular- and macromolecular drugs.

First, although typical ARDS did not show benefit from SRT in early clinical trials, the resemblance of the clinical manifestations of CARDS and NRDS rationalizes its re-investigation. Indeed, NRDS is treatable with SRT, reflected by strongly reduced mortality rates and need for oxygenation. However, besides the importance to identify COVID-19 patients that might benefit from this treatment strategy, simply reclaiming the surfactant preparations or doses applied for NRDS will likely not suffice. For example, additional research is needed regarding the optimal composition of surfactant preparations to assure resistance to inactivating agents, where further advances in the development of synthetic surfactants and the addition of non-adsorbing proteins or polymers could prove beneficial. Another important aspect is finding a substitute for the intratracheal delivery route, *e.g.* non-invasive surfactant delivery *via* aerosolization, where a balance must be found between patient comfort and protecting the personnel from infection.

Secondly, as the lungs are the main target for SARS-CoV-2 infection, inhalation of COVID-19-targeting drugs (*e.g.* (repurposed) antivirals, steroids and antibody(−based) therapeutics) is an attractive administration route. However, especially in severely ill COVID-19 patients it is challenging to target the more distal airways upon inhalation since these patients experience hypoxic and collapsed lung regions. Here, due to its excellent surface-spreading properties, pulmonary surfactant can serve as a vehicle or carrier for COVID-19-targeting drugs. This approach can be regarded as a combination therapy where the administration of surfactant can both regenerate the affected endogenous surfactant pool as well as improve drug spreading and adsorption along the respiratory surface and aid active drug targeting to specific cell types.

Lastly, the deployment of siRNA-based antivirals can alleviate the challenges associated with small molecular antivirals as this therapy is

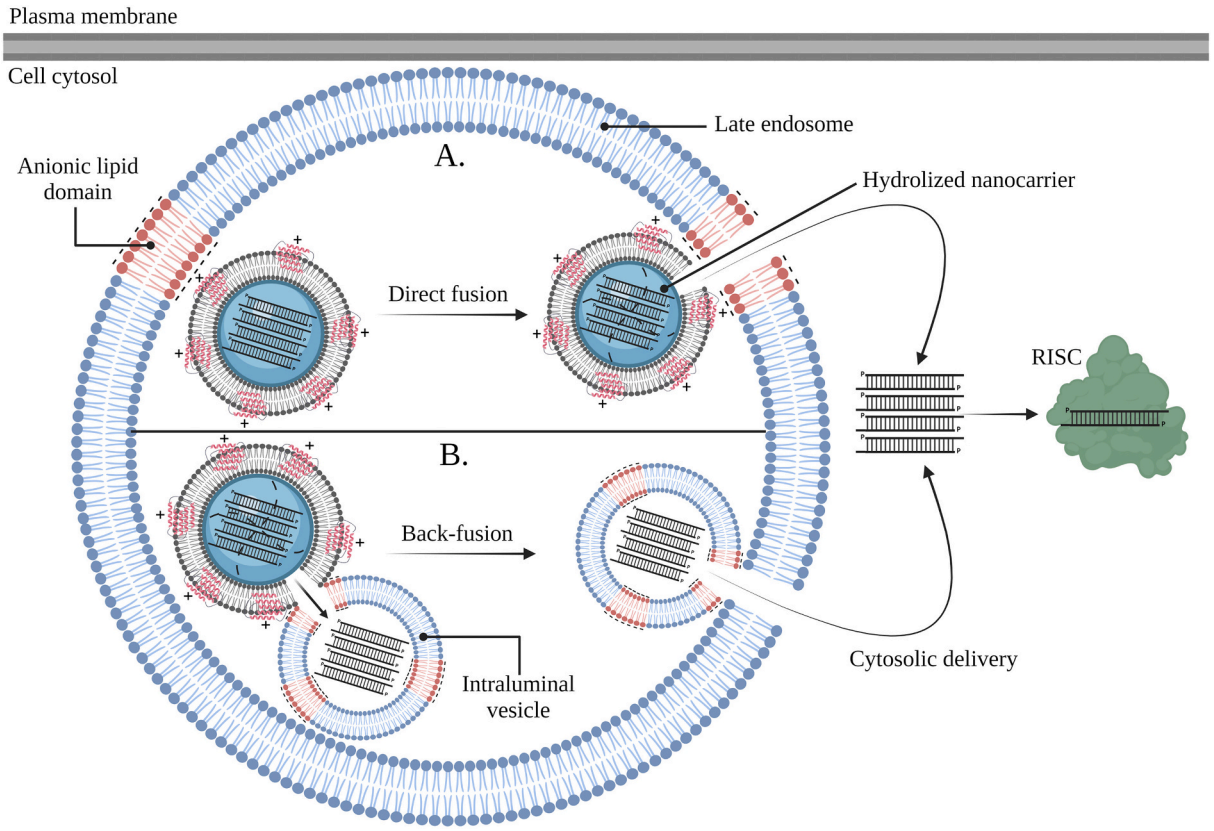

Fig. 7. SP-B-mediated cytosolic delivery of siRNA molecules occurs *via* the promotion of fusion events. The surfactant-coated siNGs are internalized by endocytosis and reside in the late endosomal compartment upon endosomal maturation. The cationic SP-B can interact with anionic lipids in the membrane of late endosomes, followed by direct fusion of the surfactant-coated siNGs with the endosomal membrane, hydrolysis of the NGs and cytosolic delivery of the siRNA molecules **(A)**. The cationic SP-B can interact with anionic lipids in intraluminal vesicles (ILVs), followed by fusion between surfactant-coated siNGs and ILVs, hydrolysis of the NGs, translocation of the siRNA molecules and cytosolic delivery upon back-fusion between the ILVs and the endosomal membrane **(B)**. Abbreviations: RISC; RNA-induced silencing complex. Created with BioRender.com

highly specific, allows to target different genes of interest simultaneously and is able to inhibit otherwise undruggable targets. To guarantee target cell entry and protection of the siRNA payload from degradation, these molecules are generally formulated into nanosized carriers. Unfortunately, to date no nanoformulation is available that allows safe and efficient siRNA delivery following inhalation. In response to this unmet need, Raemdonck and co-workers disclosed the ability of the pulmonary surfactant-associated SP-B to enhance the cytosolic delivery of siRNA. Given its endogenous nature, pulmonary surfactant-based nanoparticles could pave the way to the development of biocompatible surfactant-inspired formulations that enable efficient delivery of antiviral siRNAs to the lungs of COVID-19 patients. Moreover, it is anticipated that such formulations could be more widely used for treatment of other respiratory pathologies as well.

Declaration of Competing Interest

There are no conflicts of interest to disclose.

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