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Neutrophils in the initiation and resolution of acute pulmonary inflammation: understanding biological function and therapeutic potential

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Running Title: The role of neutrophils in ARDS

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Abstract

Acute respiratory distress syndrome (ARDS) is the often fatal sequelae of a broad range of precipitating conditions. Despite decades of intensive research and clinical trials there remain no therapies in routine clinical practice that target the dysregulated and overwhelming inflammatory response that characterises ARDS. Neutrophils play a central role in the initiation, propagation and resolution of this complex inflammatory environment by migrating into the lung and executing a variety of pro-inflammatory functions. These include degranulation with liberation of bactericidal proteins, release of cytokines and reactive oxygen species as well as production of neutrophil extracellular traps. While these functions are advantageous in clearing bacterial infection, the consequence of associated tissue damage, the contribution to worsening acute inflammation and prolonged neutrophil lifespan at sites of inflammation are deleterious. In this review, the importance of the neutrophil will be considered along with discussion of recent advances in understanding neutrophil function and the factors that influence them throughout the phases of inflammation in ARDS. From better understanding of neutrophils in this context potential therapeutic targets are identified and discussed.

Keywords

Neutrophil, ARDS, apoptosis, inflammation, neutrophil extracellular trap, chemokine, interleukin, leukotriene, DAMP, PAMP, toll-like receptor, reactive oxygen species
Introduction

Acute respiratory distress syndrome (ARDS) is the often fatal final sequelae to a broad range of direct and indirect pulmonary insults that include both infective and sterile aetiologies such as pneumonia, aspiration of gastric contents, sepsis, acute hepatic failure and acute pancreatitis. ARDS is defined by an acute onset of respiratory symptoms; profound systemic hypoxaemia; diffuse, bilateral infiltrates on chest x-ray and the exclusion of cardiac failure or fluid overload as a precipitant [1]. Despite decades of intensive research, the mortality rate for ARDS remains approximately 40% with no effective pharmacological therapies in routine clinical practice [2]. The failure to translate a large number of promising therapeutic agents from pre-clinical studies is well described [3]. Challenges arise when attempting to develop drugs that span the diverse and heterogenous conditions that precipitate ARDS, the differences in the inflammatory phenotypes and underlying genomic variation within this patient population, as well as the difficulties in the translation of observations in animal models into human inflammatory disease [3]. Distinct from inter-individual variation is also the complexity of redundancy and dysregulation of the inflammatory environment that characterises ARDS. Despite these challenges the need to develop novel therapeutics is pressing.

ARDS is characterised by an overwhelming, dysregulated and self-perpetuating pro-inflammatory environment; there is a significant increase in a range of pro-inflammatory mediators accompanied by rapid recruitment of neutrophils into the alveolar space, endothelial injury and dysfunction, platelet aggregation and microthrombus formation, interstitial and alveolar oedema, alveolar epithelial cell death and macrophage activation [4]. Diffuse alveolar damage is the typical histological hallmark of this exudative phase, although...
the histological appearances can be very variable between individuals who have died from severe ARDS [5]. Following alveolar damage, there is a proliferative phase with resolution of pulmonary oedema, type II alveolar cell hyperplasia, early collagen deposition and release of pro-resolving mediators including lipoxins and resolvins [6,7]. Whilst inflammation and injury completely resolves to leave no clinical, radiological or physiological impairment in some individuals, there remains a substantial cohort who subsequently develop diffuse pulmonary fibrosis and chronic lung disease [8].

Within this inflammatory milieu there are multiple cell types with direct roles in disease pathogenesis including macrophages, epithelial and endothelial cells. There is, however, an established body of literature that implicates the neutrophil as central to driving this inflammatory state [9,10]. Increased neutrophil numbers, the presence of neutrophil-derived proteases and the chemotactic factors that drive neutrophil recruitment are associated with increased disease severity and higher mortality rates [9,11]. Similarly, neutrophil depletion, inhibition of key chemokines and signalling molecules, or acceleration of apoptosis to shorten neutrophil lifespan, results in improvement in oxygenation, reduction in inflammation and accelerated inflammation resolution in pre-clinical models [12–15]. To date, however, clinical trials targeting neutrophil function in ARDS have failed to show benefit in overall survival [3,16].

While much has been written on the detailed mechanisms of neutrophil migration and function in inflammation [17–19] this review focuses on those observations that have been demonstrated within the context of ARDS and pre-clinical models of acute lung injury. In
doing so we hope to provide a focus on those pathological mechanisms that are of potential clinical relevance and may therefore represent therapeutic targets of the future (Table 1).

**Neutrophil recruitment and function**

The recruitment of neutrophils to the lung makes them a key factor in the pathogenesis of ARDS. In response to inflammatory mediators, either originating from the lung or distant organ injury, circulating neutrophils become primed and alter their cytoskeletal architecture with retention in the pulmonary capillary bed. They then migrate out of postcapillary venules across the endothelium, through the interstitium and epithelium and into alveoli, with associated local tissue dysfunction and destruction due to release of histotoxic mediators such as neutrophil extracellular traps, reactive oxygen species and proteases (Figure 1). This induction of epithelial and endothelial injury contributes to the development of alveolar oedema and hypoxaemia as well as exacerbating the pro-inflammatory state. It should be recognised that neutrophil migration into the lung without concomitant activation does not induce tissue injury [13]. However, there are conflicting models with regards to the mechanisms by which initial neutrophil activation occurs. It has been proposed that activation of the intravascular immune system, through an increase in circulating proinflammatory mediators, results in neutrophil priming, adhesion and/or trapping in lung capillaries. Subsequent migration along a variety of chemotactic gradients into the lung parenchyma therefore results in secondary lung injury [20]. The alternative hypothesis is that release of pro-inflammatory mediators by alveolar macrophages plays a vital role in the initiation of inflammation [21], triggering an inflammatory cascade by activating surrounding tissues and resulting in chemotaxis of inflammatory cells such as neutrophils to the airways [21]. It is likely that the exact mode of initial neutrophil recruitment and activation varies depending on
the inciting stimulus and whether this is intrapulmonary or systemic. However, the end result in both cases is the recruitment of neutrophils to the lung resulting in tissue injury.

PAMPs and DAMPs

Both sterile and infective tissue injury result in neutrophil recruitment into the lung through complementary mechanisms. In the context of infection, pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide (LPS), lipoteichoic acid, DNA, RNA and proteins such as formylated peptides are released and recognised by the immune system [22]. PAMPs can bind to, and are sensed by, a variety of pathogen recognition receptors (PRRs) including toll-like receptors (TLRs) and Nod-like receptors (NLRs) [23]. PRRs and their downstream signalling pathways drive chemotaxis as well as priming and activating both intravascular and transmigrated neutrophils in order to fulfil their bactericidal functions [22,24]. TLRs play an important role in regulating the response to pro-inflammatory mediators and are rapidly upregulated in mouse models of sepsis-related acute lung injury [25]. In early sepsis-related ARDS, downregulation of TLR1, TLR4 and TLR5 transcripts in mononuclear cells correlates with increased survival [23], while in a pulmonary contusion mouse model of lung injury, alveolar neutrophil recruitment is TLR4/MyD88 dependent [26]. Similarly, TLR4-deficiency is associated with a reduction in sterile pulmonary inflammation and more rapid resolution of injury through alterations in downstream synthesis of cysteinyl leukotrienes and subsequent induction of suppressor of cytokine signalling of cytokines 3 (SOCS3) within the lung. In this context, a reduction in TLR4-mediated oxidative stress was observed but, surprisingly, alveolar neutrophil numbers were increased [27]. While this suggests an important role for TLRs in acute lung injury, the functional importance of
neutrophils in this model is limited, thereby serving to emphasise that understanding of neutrophil-mediated TLR function in ARDS requires further investigation.

Sterile tissue injury, either in the context of direct injury to lung parenchyma or distant organ injury, results in necrotic cell death with the release of a range of damage-associated molecular patterns (DAMPs) into the extracellular environment. These DAMPs serve to induce a proinflammatory response which drives neutrophil recruitment into the lung [25,27]. A number of DAMPs have been described in ARDS including high mobility group box 1 (HMGB1), heat shock proteins 60 and 72, hyaluronan and a range of mitochondrial-derived factors including DNA, formylated peptides and cardiolipin [28]. Due to common evolutionary ancestry and relative structural and sequence homology with bacteria it appears these mitochondrial factors play an important role in driving the development of neutrophil-mediated lung injury. Mitochondrial DNA is elevated in patients with ARDS and, through interaction with endosomal TLR9, mediates neutrophil recruitment [29]. Importantly, it is also been shown to be a predictive biomarker of mortality in patients in intensive care, including those with ARDS, and therefore further studies with regards to both its role as a clinically significant predictive biomarker in ARDS and as a therapeutic target are needed.

Mitochondrial formylated peptides play a crucial role in neutrophil recruitment in ARDS as well as altering epithelial and endothelial cell function [29–31]. Elevated levels of these peptides are found in bronchoalveolar lavage fluid and serum of ARDS patients [31]. The importance of formyl peptide receptor 1 (FPR1, the cognate receptor for formylated peptides) in influencing acute inflammation is well established [32]. Genetic deletion of Fpr1 in mice is associated with reduced survival in infection but an attenuated inflammatory response in

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the context of sterile tissue injury [29,31,33]. FPR1, a G-protein coupled receptor, activates a variety of intracellular signalling pathways including PI3K, mitogen-activated protein kinases (MAPKs) and Akt pathways [34]. This serves to directly alter neutrophil migration, reactive oxygen species production, degranulation and transcriptional activity [32]. In sterile lung injury in mice, neutrophil chemotaxis, along with other indices of pulmonary inflammation, were diminished in $Fpr1^{-/-}$ mice or in the presence of an FPR1 antagonist delivered either prior to, or following, acid-induced lung injury. This suggests that FPR1 may represent a therapeutic target in sterile ARDS but, as with many other therapies targeting neutrophil function, addressing the challenge of concurrent infection needs to be addressed [31].

**Chemokines and Cytokines**

Chemokines, a family of chemotactic cytokines, play a crucial role in neutrophil migration to sites of inflammation [35]. CXC chemokines, in particular CXCL8 (IL-8), play an important role in neutrophil chemotaxis in ARDS with elevated levels associated with poor disease prognosis, increased severity and mortality [36–38]. Produced by local immune cells and epithelial cells, CXCL8 is not the only chemokine responsible for the recruitment of neutrophils to the lung, as blockade results in only partial reduction in alveolar neutrophil number [13,35]. CCL2 and CCL7 are also elevated in the BALF of both LPS-challenged volunteers and ARDS patients and neutralising either chemokine reduces neutrophil chemotactic responses in vitro [39]. Interestingly, CCL2 and CCL7 also potentiate the activity of CXCL8, suggesting synergistic activity between these chemokines drives neutrophil recruitment in ARDS. The CXCL8 receptor, CXCR1, is more highly expressed on circulating neutrophils from ARDS patients relative to the CCL2/7 receptors CCR1, CCR2 and CCR3 [39]. However, a significant increase in neutrophil CCR2 expression in BALF

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fluids has been observed, with the authors postulating that this confers an increased sensitivity to cognate ligands CCL2 and CCL7 in the alveolar space and therefore suggesting an important role in neutrophil chemotaxis within the lung. Other chemokines including CXCL5, and mediators such as C5a and leukotriene B₄ (LTB₄), have also been shown to have a role in driving neutrophil chemotaxis in ARDS [40].

While the majority of cytokines are produced by other cell types, neutrophils also secrete a range of cytokines that potentiate the inflammatory response. These include TNF, which has been associated with microvascular plasma protein leakage [41], and IL-1β, which potentiates the pro-inflammatory cycle by inducing further cytokine and chemokine release and thereby recruiting more neutrophils to the lung [42]. Furthermore, antibody-mediated inhibition of the TNF receptor (TNFR1) reduced alveolar neutrophil recruitment, inflammatory cytokine release and biomarkers of endothelial injury in BALF and serum samples in experimental acute lung injury. As a result, inhibiting TNFR1 could be considered as a potential option in the treatment of ARDS [43].

After a non-pulmonary acute injury such as traumatic brain injury, burn injury or sepsis, mediators including IL-1β, IL-6, CXCL8, IL-18 and TNF as well as a variety of DAMPs are released into the systemic circulation [23,36,44–47]. For example, intravascular neutrophil priming and activation, as part of a systemic inflammatory response syndrome that occurs following traumatic brain injury, results in neutrophil migration into the lung [47] and other organs including the liver and kidney [48,49], inducing tissue injury and dysfunction. Similarly, the pro-inflammatory cytokines TNF and IL-1β were found to be elevated in the BALF of ARDS patients alongside the natural antagonists IL-1RA and soluble TNF receptors.
It appears, however, that an imbalance between agonists and antagonists exists which drives the acute phase inflammatory response [51].

At present, there have been no specific clinical trials investigating the pharmacological manipulation of the majority of chemokines, although steroids effecting their function through suppression of chemokine/cytokine axis has been proposed. Current clinical trial data on the use of steroids in ARDS is mixed and there is no definitive evidence for improved survival, and in some cases has been found to worsen outcome. While improved effects on ventilator-free days has been described, an increase in return to mechanical ventilation is increased amongst those receiving steroids, as are significant side effects including neuromyopathy and hyperglycaemia) [52–55].

Selectins and Integrins

In order for neutrophils to enter the alveolar space, selectins play an important role in initiating the process of neutrophil tethering and rolling along the endothelial surface. L-selectin, one such adhesion molecule present on neutrophils, has been found (in its soluble form) to be reduced in the plasma of ARDS patients, which directly correlated to ventilation requirements, degree of respiratory failure and mortality [57,58]. Conversely, elevated plasma levels of E-selectin and P-selectin, expressed by endothelial cells, correlated with increased mortality [58–60]. Most recently, through genome wide association studies, three nonsynonymous single nucleotide polymorphism (SNPs) in the Selectin P Ligand Gene (SELPLG) have been identified to be associated with sepsis-related ARDS [61]. PSGL1 (the encoded protein) acts as an important ligand for both L-selectin and P-selectin. In LPS-induced lung injury, inflammation is attenuated in Selplg−/− mice while an inhibitory antibody
to PSGL-1 also limits inflammation in LPS- and ventilator-induced lung injury models [61]. The exact mechanisms through which the SELPLG SNPs exert their functional effect is not known it was postulated that alteration in amino acid sequence may result in altered P-selectin binding affinity and therefore alter neutrophil rolling [61].

Once tethering and rolling are initiated, integrins play a role in slowing down and immobilising neutrophils to allow transendothelial migration and activation [62]. Surprisingly, neutralising antibodies to the β2 integrin CD18 in the context of sterile lung inflammation results in increased alveolar neutrophils but a reduction in neutrophil-mediated pulmonary injury, suggesting that its predominant role is in neutrophil activation rather than chemotaxis [13]. β2 integrins on the surface of activated neutrophils induce heparin-binding protein (HBP) release through phosphoinositide 3-kinase (PI3K)-dependent signalling [63]. Antibody-mediated blockade of β2 integrin function resulted in lower levels of circulating HBP and a subsequent reduction in pulmonary oedema, which the authors propose is principally through a reduction in vascular leak and endothelial dysfunction [46]. The β2 integrin binds to intracellular adhesion molecule 1 (ICAM-1) on endothelial cells to aid in neutrophil transmigration [64]. Soluble ICAM-1 is elevated in ARDS patients and its inhibition reduces sterile lung injury in mice [65,66].

**NETosis**

NETosis, the process through which neutrophils release extracellular DNA in order to trap and contain bacteria, is an important defence mechanism against invading pathogens. Increased NET production has recently been associated with increased ARDS severity [67,68]. Lefrançais et al. demonstrated that circulating neutrophils from ARDS patients
produce significantly more NETs upon phorbol myristate acetate stimulation than those from healthy donors [68]. As NETs contain and can release neutrophil elastases, myeloperoxidase, DNA and histones, they can also potentiate the tissue damage observed in ARDS, in part through cytotoxic effects on epithelial and endothelial cells [69]. Reducing NETs either by intratracheal DNase I treatment or the partial deficiency of protein arginine deiminase 4 (PAD4+/−; a protein involved in the projection of NETs) increased survival in a mouse model of severe bacterial pneumonia/acute lung injury [68]. Although partial deficiency of PAD4 reduces lung injury, complete knock out increases bacterial burden. This suggests that a NET balance is necessary and that the potential deleterious or beneficial effects of NETs in ARDS may relate to the presence of microbial infection [68]. Furthermore, a phase III clinical trial is currently investigating the effectiveness of inhaled dornase alpha, recombinant human DNase 1, in reducing the incidence of moderate to severe ARDS in severe trauma patients through accelerated degradation of extracellular DNA, including NETs (Table 1) [70].

Granule proteins

The release of various granule proteins including elastases, matrix metalloproteinases (MMP) and cationic polypeptides have been associated with the propagation of ARDS [71]. Neutrophil elastases (NE) are implicated in lung injury, although it is unclear whether the damage is principally to endothelial or epithelial cells or as a result of degradation of the alveolar basement membrane [72,73]. Plasma levels of NE and the endogenous proteinase inhibitor elafin are predictors of ARDS mortality [74], while inhibition of NE reduces lung injury in various animal models [75–79]. Mice deficient in NE are more susceptible to gram-negative bacteria, suggesting that NE is required for adequate host defence against invading pathogens complete inhibition of NE can be harmful [80]. Despite data from pre-clinical
models, sivelestat, a selective NE-inhibitor, does not alter 28-day mortality in a number of clinical trials (Table 1) [81]. Although alteration in oxygenation has been observed the small sample sizes of the majority of clinical trials and heterogenous patient populations potentially mask any benefit in sub-groups of patients with ARDS [81]. It is difficult to separate challenges in clinical trial design from limitations of biological importance in this context, therefore further study is required to clarify both aspects of this problem.

MMPs are zinc-dependant endopeptidases with numerous biological functions such as tissue remodelling, wound healing and angiogenesis [82]. Fligiel and colleagues investigated numerous MMPs and their natural antagonists tissue inhibitor of metalloproteinases (TIMPs) in BALF fluids of ARDS patients [83]. MMP-2, MMP-8 and MMP-9 are proteases secreted by neutrophils and their levels were elevated in all patients along with neutrophil number. Furthermore, elevated MMP-1 and MMP-3 was associated with increased mortality [83]. Although neutrophils do not produce MMP-1 and MMP-3, they can induce MMP-1 secretion in human vascular smooth muscle cells which in turn acts in an autocrine feedback loop to produce CXCL8 and induce neutrophil chemotaxis [84]. Consistent with this, MMP-3 deficient mice have reduced neutrophil migration into the lung and attenuated neutrophil-mediated epithelial and vascular damage in the context of immune complex-mediated pulmonary injury [85]. MMP-13 has shown to be play a role in the development of sepsis-mediated acute lung injury [86]. Hypertonic saline has shown to reduce the production of MMPs such as MMP-9 and MMP-13 in mouse models of acute lung injury, thereby reducing disease progression and inflammation; an open-label clinical trial is currently underway to evaluate efficacy in post-traumatic acute lung injury (Table 1) [86–88].
As mentioned previously, HBP is a cationic peptide that plays an important role in neutrophil-mediated vascular leakage through increased endothelial permeability [89]. In trauma patients admitted to intensive care, early elevation of HBP after admission was a predictor for the development of ARDS suggesting that HBP may be a potential biomarker for the early detection of ARDS although further work is required [90]. Plasma HBP has also been shown to be an independent predictor for 30-day mortality in ARDS [91]. Administration of simvastatin to patients with acute lung injury reduced HBP plasma levels [92]. Simvastatin did not improve overall survival in ARDS patients, however secondary analysis has identified improvement in 28- and 90-day survival in patients with a hyperinflammatory subphenotype relative to placebo control (Table 1) [93].

Defensins are arginine-rich cationic proteins which have antimicrobial properties [94]. Divided into two subgroups, α-defensins and β-defensins exhibit different roles. Neutrophils store α-defensins in their granules and release them in attempt to eradicate microbes, with β-defensins primarily expressed by mucosal surface epithelial cells. However, defensins can also result in tissue damage, as observed in ARDS [95]. Elevated levels of α-defensins were found in BALF of ARDS patients and higher levels correlate with increased severity of lung injury [95]. Although plasma α-defensin was also elevated it did not correlate with prognosis - it has been proposed that circulating α-defensin originates from the bone-marrow rather than directly from neutrophils and therefore have different functional effects in this context. Although not known to be produced by neutrophils, β-defensins are implicated in the pathogenesis of ARDS. β-defensin-3 inhibits neutrophil apoptosis by downregulating Bid, a pro-apoptotic protein, and upregulating the anti-apoptotic protein Bcl-xL in neutrophils [96]. This delay in apoptosis is dependent upon interaction of β-defensin-3 with the chemokine receptor CCR6, with the effect attenuated in the presence of a CCR6-specific blocking
antibody [96]. As discussed below, delay in neutrophil apoptosis is associated with an increased severity in lung injury.

LL-37 is another cationic protein with antimicrobial properties released from neutrophil granules [97]. It also carries the ability to activate neutrophils and augment the inflammatory cascade [98] and LL-37 is elevated in BALF samples of ARDS patients relative to healthy volunteers [97]. Interestingly, although elevated LL-37 correlated with increased lung injury, LL-37 did not correlate with neutrophil counts, suggesting that neutrophils are not the only source of LL-37 with macrophages and epithelial cell production also described [97].

**Reactive oxygen species**

Reactive oxygen species (ROS) play an important role in eliminating pathogens within phagosomes and for the generation of NETs but also act as chemoattractants for immune cells resulting in tissue repair [99]. However, excess ROS production results in oxidative stress and plays a major role in lung damage through the release of pro-inflammatory cytokines, enhanced recruitment of immune cells and consequently the progression of ARDS [99]. Neutrophils have been shown to produce ROS when activated and contribute to oxidative stress [99]. Furthermore, increased permeability of the endothelial and epithelial barrier is observed, increasing neutrophil transmigration to the alveolar space [99]. Additionally, an increase in oxidised molecules and reduction in anti-oxidant proteins is observed in BALF fluid of ARDS patients which serves to perpetuate lung damage [100]. Glutathione plays a vital role in neutralising hydrogen peroxide, a major contributor to oxidative damage, through the enzyme glutathione peroxidase by converting glutathione to glutathione disulphide [101]. Administration of the anti-oxidant N-acetylcysteine restores the

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oxidant balance by increasing glutathione levels in erythrocytes. Several clinical trials have investigated the role of N-acetylcysteine as a therapeutic strategy in ARDS with variable results. A recent meta-analysis concluded that, although duration of intensive care admission is shortened, there is no demonstrable effect on overall outcome or 30-day survival [102].

Mechanisms of cell death

In addition to marked inflammatory cell activation and recruitment, the pathogenesis of ARDS is characterized by alterations in a variety of forms of cell death. Death and damage to the alveolar epithelial and alveolar endothelial cells is thought to play a key role in the initiation and progression of the disease process [103,104], while inflammatory cell apoptosis and subsequent clearance is an important step in inflammation resolution [105,106]. While apoptosis (described further below) is undoubtedly the most studied form of cell death, there has also been a recent ‘-osis explosion’ with increased knowledge of alternative cell death pathways such as pyroptosis, necroptosis, ferroptosis, entosis and NETosis [107]. While some of these non-apoptotic pathways are likely to have relevance to the pathogenesis of ARDS, this is an as yet understudied area that will hopefully lead to future novel avenues for therapeutic intervention.

Targeting apoptosis

Apoptosis occurs through two distinct but converging pathways. The intrinsic pathway is activated in response to diverse stimuli including DNA damage, ROS exposure and endoplasmic reticulum stress. The central event in intrinsic apoptosis is mitochondrial outer membrane permeabilization that allows escape of pro-apoptotic molecules such as cytochrome-c which then form a caspase-activating complex. Active caspases act as the
executioners of apoptosis leading to cellular disassembly of the cell, DNA degradation, cell surface phosphatidylserine exposure and pannexin channel activation, all hallmarks of apoptotic cell death. Mitochondrial outer membrane permeabilization is itself controlled by intracellular Bcl2 family proteins which includes both pro- and anti-apoptotic members (such as Bid and Bcl-xL, described above). In contrast, extrinsic apoptosis is usually activated by a cell surface death receptor upon interaction with its cognate ligand which then leads to caspase activation. The multiple steps and checkpoints involved in apoptotic cell death both allow this to be dysregulated at multiple steps in human diseases such as ARDS, but also allows the potential for therapeutic intervention at several levels [108].

Neutrophil apoptosis in ARDS has been shown to be delayed by several groups including our own [109–112] and correlates with disease severity in sepsis and sepsis-related ARDS. Interestingly, BALF from patients with early ARDS (days 1 and 3 of disease) but not late ARDS directly inhibits apoptosis of healthy donor neutrophils [109]. This effect has been attributed to soluble factors including GM-CSF, G-CSF, CXCL8 and IL-2 [109,113]. Recent detailed phenotyping of ARDS neutrophils has revealed multiple phenotypic alterations alongside delayed apoptosis [112]. Interestingly, ARDS BALF-induced delay of healthy neutrophil apoptosis could be overcome by PI3K inhibition, whereas the anti-apoptosis phenotype of ARDS patient neutrophils was resistant to PI3K inhibition [112]. This suggests that additional PI3K-independent mechanisms are in play within the complex pro-inflammatory milieu experienced during human ARDS.

Several other pre-clinical strategies targeting neutrophil apoptosis have also shown promise in the treatment of lung injury. Targeting of the extrinsic pathway of apoptosis has been
achieved by TRAIL (TNF-related apoptosis-inducing ligand), part of the TNF family of ligands that can initiate apoptosis by activating cell surface receptors [114]. TRAIL appears to have no role in constitutive neutrophil apoptosis nor neutrophil chemotaxis (in contrast to the TNF family ligand FasL which is a potent neutrophil chemoattractant). However, in response to LPS-induced lung injury, TRAIL acts to limit inflammation and enhances neutrophil apoptosis [115]. Furthermore, recombinant TRAIL was able to induce an anti-inflammatory response, suggesting such strategies may have therapeutic potential in human ARDS. Targeting of the intrinsic pathway of neutrophil apoptosis, such as with cyclin-dependent kinase inhibitor (CDKi) drugs, has also been shown to have potent anti-inflammatory effects in animal models of neutrophil dominant inflammation [14,15]. CDKi drugs principally induce neutrophil apoptosis by inhibiting CDK9-mediated transcription of the short lived Bcl2 member Mcl-1 [14,116]. As neutrophils have limited expression of the main anti-apoptotic Bcl2 homologue, Bcl2 itself, this leaves them sensitive to alterations in Mcl-1 leading to apoptosis. CDKi drugs enhance resolution of several lung injury models including bleomycin-induced, endotoxin-induced and bacteria-induced lung injury [14,117]. Interestingly, in an *Escherichia coli*-induced model of acute lung injury, a CDKi drug administered after the onset of inflammation augmented the resolution of lung inflammation without detrimentally reducing clearance of the bacteria. Indeed, there was increased bacterial clearance possibly resulting from lipid-mediated enhanced bacterial phagocytosis by macrophages [14]. Furthermore, and in contrast to that observed with PI3K inhibition [112], CDKi has recently been shown to override the delayed neutrophil apoptosis in sepsis-induced human ARDS concurrent with reduced expression of Mcl-1 [111]. This suggests that Mcl-1 targeting approaches (either with CDKi or with use of novel small molecule Mcl-1 inhibitors) may have therapeutic potential in human ARDS.
Other strategies to modulate neutrophil apoptosis warrant further investigation in the context of ARDS. Potential strategies include the use of a p21 (Cdkn1a) peptide which binds and sequesters proliferating cell nuclear antigen (PCNA), an important endogenous neutrophil anti-apoptotic factor [118]. While p21 peptide is able to induce apoptosis of neutrophils isolated from patients with lung inflammation [119], testing in in vivo models of ARDS is awaited. Several families of anti-inflammatory lipid mediators which influence neutrophil lifespan and their clearance (amongst other pleiotropic effects) have also been delineated. These include the lipids 15-epi-lipoxin A4 and resolvin E1 which drive neutrophil apoptosis and attenuate experimental lung injury [120,121].

**Lung parenchymal cell death**

In contrast to the potential benefits of inflammatory cell apoptosis during lung injury, there is also evidence that damage and death of the lung epithelium and endothelium can contribute to disease pathogenesis [109]. Alveolar epithelial apoptosis is observed in experimental lung injury caused by bleomycin, endotoxin and acid [31,122], while alterations in Bcl2 members (including increases in pro-apoptotic Bax) have been observed in lung epithelium from human lung injury cases [123]. In addition, BALF from human ARDS patients contains FasL which activates Fas receptor to induce extrinsic pathway apoptosis [124]. Lung epithelium expresses Fas, with ARDS BALF able to induce epithelial apoptosis in a Fas/FasL-dependent fashion [124], but abolished Fas activity was unable to protect in experimental virus-induced ARDS [125]. Further work is needed to clarify the role and timing of Fas/FasL strategies, especially as Fas can also influence macrophage dynamics during resolution of lung injury [126].
Similarly, death of pulmonary endothelium has recently been demonstrated to be a pathogenic response in endotoxin-induced lung injury [127]. This elegant study demonstrated that endotoxin exposure led to activation of caspase-4/5 (caspase-11 in mice) in endothelium and a consequent pro-inflammatory, lytic form of cell death (termed pyroptosis). Conditional deletion of caspase-11 specifically in endothelial cells (using a Cre/lox system) led to reduced endotoxin-induced lung oedema, neutrophil accumulation and death. Caspase-11 inhibitors such as wedololactone suppress endotoxin-induced caspase-11 in vitro [128] but their role in lung injury in vivo remains to be tested. In summary, any potential anti-inflammatory strategy based on modulation of inflammatory cell death has to carefully balance potentially deleterious off-target effects should cell death be induced in lung parenchymal cells.

**Clearance of apoptotic cells**

Macrophages play a crucial role in limiting excessive inflammation and augmenting tissue repair, not only in the clearance of apoptotic and necrotic cells, but also removal of neutrophils undergoing NETosis [129,130]. In ARDS, however, macrophage phagocytic function is impaired [130]. Grégoire and colleagues observed enhanced NET formation and reduced neutrophil apoptosis coupled with a reduction in macrophage clearance of apoptotic cells (efferocytosis) in BALF from ARDS patients [130]. AMP-activated protein kinase (AMPK) has been associated with increasing macrophage phagocytosis and reduced TNF and IL-6 production [131]. The addition of metformin, an AMPK activator, to ARDS BALF samples resulted in removal of NETs and increased efferocytosis by macrophages [130]. Additionally, AMPK activators administered in an LPS-induced mouse lung injury model reduced alveolar neutrophil accumulation, pulmonary oedema and BALF TNF and IL-6. Furthermore, retrospective analysis of diabetic patients on metformin for the three months

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prior to developing ARDS had a non-significant reduction in 30-day mortality from 55.32% to 42.42%. Little is known about the exact anti-inflammatory mechanism of metformin in this context and therefore requires further study [132].

A similar observation was made using a neutralising antibody against HMGB1 to increase macrophage efferocytosis [130]. HMGB1, which is increased in ARDS [133], inhibits efferocytosis by interfering with the binding between the phosphatidylserine bridging molecule milk fat globule EGF factor 8 (MFG-E8) and the αvβ3 integrin on the surface of macrophages [134]. Importantly, MFG-E8 knockout mice have increased apoptotic alveolar neutrophils in the alveolar space following LPS-induced injury, an effect that can be rescued by recombinant MFG-E8 [135].

Another potential mechanism which some have speculated may be involved in neutrophil clearance is the relatively recently described concept of reverse migration. This is the process by which neutrophils migrate in the opposite direction to the chemotactic gradients that initially recruited them [136,137]. As yet this process has only been demonstrated in zebrafish and mouse models, with no firm data demonstrating a direct role in human disease. In animal models, prostaglandin E2 (PGE$_2$) is an important mediator of neutrophil reverse migration with macrophage depletion resulting in inhibited reverse migration and therefore delayed resolution of inflammation due to reduced PGE$_2$ production [138]. Additionally, PGE$_2$ depletion has a similar effect, further validating its importance in resolution. PGE$_2$ signals through the EP4 receptor, increasing Alox12 production and consequently lipoxin A$_4$, an important pro-resolving mediator which enhances reverse migration [138]. In the context of resolution of pulmonary inflammation in mice, LTB$_4$ released by neutrophils promotes

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neutrophil elastase release, which subsequently cleaves junctional adhesion molecule-C (JAM-C) from the endothelium of post-capillary venules facilitating reverse migration of neutrophils [139]. Although the departure of neutrophils from sites of inflammation can be considered a sign of inflammatory resolution, reverse migration also has the potential to propagate inflammation. Local ischaemia-reperfusion to the ear skin or cremaster muscle in mice can progress to a systemic inflammatory response in numerous organs including the lung, heart and liver [139]. Pharmacological or genetic interference to either enhance or inhibit reverse migration led to a parallel increase or decrease in secondary inflammation in the lung distant organs [139]. In humans, increased levels of soluble JAM-C were detected in the plasma of ARDS patients, hinting that reverse migration may be occurring with a significant direct correlation observed between soluble JAM-C and the severity of multi-organ failure [139]. These data therefore suggest that neutrophil reverse migration may not be simply a clearance mechanism but has the potential to cause dysregulated systemic pro-inflammation in ARDS.

**Conclusion**

Neutrophils are both a hallmark and driver of ARDS acting in concert with other resident and recruited inflammatory cell types to induce a dysregulated, overwhelming and often fatal pro-inflammatory state within the lung (Figure 1). To conclude that a simple “one-size fits all” approach in the context of both the pathogenesis and potential therapeutics is perhaps naive and out-dated. This conclusion is supported by the identification of distinct sub-populations of ARDS patients who respond differently to fluid management, ventilation strategies and some pharmacological therapies [93,140,141]. Recognition of these different hypo- and hyper-inflammatory phenotypes within the ARDS cohort as well as ever-increasing numbers
of predictive and prognostic biomarkers is leading to a shift in clinical trial design to reduce clinical and biological heterogeneity [3]. New combination therapies that target a variety of inflammatory components in ARDS are also being considered to address issues of redundancy, as are cell based therapies including bone marrow derived myeloid suppressor cells in infection-related ARDS [3].

It is therefore essential that variations in neutrophil phenotype and function as well as understanding of neutrophil behaviour in different patient cohorts is explored and characterised. As has been outlined, the difficulties of neutrophil-specific therapies in the context of infection-related ARDS warrants further exploration but, as in the context of induction of neutrophil apoptosis, this should not stop further investigation particularly in the context of combination therapies. Further research is also required on the complex chemokine networks, NETosis, mechanisms of inflammation resolution as well as strategies that aim to protect or enhance repair of damaged epithelial and endothelial beds. It is now over fifty years since ARDS was first described [142] and much has been learnt about its pathogenesis and beneficial supportive ventilation and fluid management strategies but the era of effective pharmacological treatments is yet to dawn. Targeting aspects of neutrophil biology is likely to have a place among those therapies.
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Author contribution

All authors contributed to manuscript writing and revision. All authors approved the final version.

List of abbreviations:

AMPK, AMP-activated protein kinase;
ARDs, acute respiratory distress syndrome;
BALF, bronchoalveolar lavage fluid;
BLT2, leukotriene B4 receptor 2;
CCL/CXCL, chemokine ligands;
CDK, cyclin dependent kinase;
CDKi, cyclin-dependent kinase inhibitor;
CXCR, chemokine receptor;
DBP, Vitamin D binding protein;
FPR, formyl peptide receptor;
G-CSF, granulocyte-colony stimulating factor;
GM-CSF, granulocyte/macrophage-stimulating factor;
HBP, heparin- binding protein;
HMGB1, high mobility group box;

IL, interleukin;

ICAM-1, intracellular adhesion molecule 1;

JAM-C, junctional adhesion molecule-C;

LPS, lipopolysaccharide;

LTB₄, leukotriene B₄;

MFG-E8, milk fat globule EGF factor 8;

MMP, matrix metalloproteinase;

NE, neutrophil elastases;

NET, neutrophil extracellular trap;

NLR, Nod-like receptor;

PGE₂, prostaglandin E₂;

PI3K, Phosphoinositide 3-kinase;

PAD4, protein arginine deiminase 4;

PAI, Plasminogen activator inhibitor;

PAMP, pathogen-associated molecular pattern;

PRR, pathogen recognition receptor;

ROS, reactive oxygen species;

SELPG, Selectin P ligand gene;

SNP, single nucleotide polymorphism;

TIMP, tissue inhibitors of metalloproteinases;

TLR, toll-like receptor;

TNF, tumour necrosis factor;

TRAIL, TNF-related apoptosis-inducing ligand
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## Table 1. Neutrophil-related mediators in ARDS, pre-clinical observations and associated therapeutics

|   | Pre-clinical observations | Human ARDS | Therapeutic potential | Therapeutics | Refs |
|---|---------------------------|------------|-----------------------|--------------|------|
| **PAMPs** | Neutrophil recruitment and activation | Unknown | TLR1, TLR4, TLR5 antagonists | Not clinically tested | [23,143] |
| **DAMPs** | Mitochondrial formylated peptides | ↑ neutrophil migration and inflammation | ↑ in blood and BALF | FPR1 antagonists | Not clinically tested | [31] |
| | Mitochondrial DNA | ↑ neutrophil migration and inflammation | ↑ in blood and BALF | TLR9 antagonists | Not clinically tested | [30] |
| | HMGB1 | ↑ neutrophils and inflammation | ↑ in blood | Metformin | No increase in survival | [132,133,144] |
| **Chemokines/ Cytokines** | CXCL5 | Neutrophil chemotaxis | ↑ in BALF | CXCL5 antibody | Not clinically tested | [145] |
| | CXCL8 | Neutrophil chemotaxis | ↑ in blood and BALF | CXCL8 antibody, CXCR1, CXCR2 antagonist | allogeneic adipose-derived mesenchymal stem cells – no effect | [36,38,146] |
| | CCL2 | Neutrophil chemotaxis | ↑ in BALF | CCL2 antibody | Not clinically tested | [39] |
| | CCL7 | Neutrophil chemotaxis | ↑ in BALF | CCL7 antibody, CCR1, CCR2, CCR3 antagonist | Not clinically tested | [39] |
| | LTB₄ | Neutrophil chemotaxis | ↑ in blood and BALF | LTB₄ antibody, BLT2 antagonist | Not clinically tested | [147,148] |
| | C5a | Neutrophil chemotaxis | ↑ in BALF | Anti-DBP (DBP=Vitamin D binding protein) | Not clinically tested | [149] |
| | TNF | Pro-inflammatory response Microvascular plasma protein leakage | ↑ in blood and BALF | TNF antibody, TNF-RA | Not clinically tested | [41,43,50,150] |
| | IL-1β | Pro-inflammatory response | ↑ in blood and BALF | IL-1β antibody | Not clinically tested | [50,150] |
| **IL-1RA** | Neutralises TNF imbalance | ↑ in BALF | Administration for TNF neutralisation | Not clinically tested | [50] |
|----------|-----------------------------|-----------|--------------------------------------|----------------------|------|
| **TNF receptor** | Neutralises IL-1β imbalance | ↑ in BALF | Administration for IL-1β neutralisation | Not clinically tested | [50] |
| **Selectins** | L-selectin | Aids in neutrophil migration | Soluble form ↓ in blood | L-selectin antibody | Not clinically tested | [57,151] |
| | E-selectin | Aids in neutrophil migration | Soluble form ↑ in blood | E-selectin antibody | Not clinically tested | [57] |
| | P-selectin | Aids in neutrophil migration | Soluble form ↑ in blood | P-selectin antibody | Not clinically tested | [57,66] |
| **Integrins** | β2 integrin | Aids in neutrophil migration | ↑ sICAM-1 in blood and BALF | β2 integrin antibody | Not clinically tested | [62,65] |
| **NETs** | L-selectin antibody | Not clinically tested | [62,65] |
| | E-selectin antibody | Not clinically tested | [57] |
| | P-selectin antibody | Not clinically tested | [57,66] |
| **NETs** | Lung injury | ↑ in BALF | DNAase I | Inhaled DNAse I – Phase III | [68,70,152] |
| **Granule proteins** | Neutrophil elastase | Lung injury | ↑ in blood and BALF | Anti-elastase therapies | No increase in survival | [72,79,153,154] |
| | Elafin | Neutrophil elastase inhibitor | ↓ in blood | Administration | Not clinically tested | [154] |
| | MMP-1 | Lung injury | ↑ in BALF | Inhibit with TIMP | Nebulised hypertonic saline- Phase I | [83,86,88,155] |
| | MMP-2 | Lung injury | ↑ in BALF | Inhibit with TIMP | Nebulised hypertonic saline- Phase I | [83,86,88,155] |
| | MMP-3 | Lung injury | ↑ in BALF | Inhibit with TIMP | Nebulised hypertonic saline- Phase I | [83,86,88,155] |
| | MMP-8 | Lung injury | ↑ in blood and BALF | Inhibit with TIMP | Nebulised hypertonic saline- Phase I | [83,86,88,155,156] |
| | MMP-9 | Lung injury | ↑ in BALF | Inhibit with TIMP | Nebulised hypertonic saline- Phase I | [83,86,88,155] |
| | TIMP-1 | Inhibits MMPs | ↑ in blood and BALF | Administration | Not clinically tested | [83,156] |
| | HBP | Vascular leakage | ↑ in blood | Simvastatin reduced serum HBP | Simvastatin ↑ survival in hyperinflammatory subphenotype | [89,90,92,93] |
| | α-defensin | Lung injury | ↑ in blood and BALF | Inhibit | Not clinically tested | [95] |
| | β-defensin | Inhibit neutrophil apoptosis | Unknown | Inhibit | Not clinically tested | [96] |
| | LL-37 | Neutrophil activation | ↑ in BALF | Inhibit | Not clinically tested | [97] |

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| Reactive oxygen species | H$_2$O$_2$ | Lung tissue damage | ↑ in breath condensate | Neutralising with antioxidants Pan-PI3K inhibitor | Inhaled carbon monoxide – Phase I | [99,112,157,158] |
|-------------------------|-----------|--------------------|------------------------|-----------------------------------------------|-------------------------------------|------------------|
|                         | H$_2$O$_2$| Release pro-inflammatory mediators Immune cells recruitment Glutathione Neutralises H$_2$O$_2$ ↑ oxidised glutathione in BALF | Intravenous administration | No effect | [101,102] |
| Apoptosis               | PAI-1     | ↓ neutrophil apoptosis | ↑ in BALF | PAI-1 antagonist | Not clinically tested | [159] |
|                         | Mcl-1     | ↓ neutrophil apoptosis | ↑ in ARDS neutrophils | CDK inhibitor | Not clinically tested | [111] |

H$_2$O$_2$, Hydrogen peroxide; CCL/CXCL, chemokine ligands; PI3K, Phosphoinositide 3-kinase; BALF, bronchoalveolar lavage fluid; CXCR, chemokine receptor; LTB$_4$, leukotriene B4; BLT2, leukotriene B4 receptor 2; DBP, Vitamin D binding protein; TNF, tumour necrosis factor; IL, interleukin; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; HBP, heparin-binding protein; FPR, formyl peptide receptor; HMGB1, high mobility group box; TLR, toll-like receptor; PAI, Plasminogen activator inhibitor; CDK, cyclin dependent kinase.
Figure legends

Figure 1. Initiation and resolution of neutrophil-mediated inflammation in ARDS. (A) The healthy alveolar unit facilitates rapid gas transfer with the presence of resident alveolar macrophages providing rapid response to pulmonary infection and injury. (B) Following infection and/or tissue injury, release of pathogen associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) directly induces neutrophil recruitment into the alveolar space in addition to a range of chemokines and mediators secreted by macrophages and epithelial cells. (C) Neutrophils exert multiple pro-inflammatory functions with the release of reactive oxygen species, proteases, neutrophil extracellular traps and cytokines as well as phagocytosis of bacteria. This is accompanied by accumulation of oedema within the alveolus, endothelial dysfunction and epithelial cell death. (D) Resolution of inflammation occurs through neutrophil apoptosis and macrophage clearance of apoptotic cells (efferocytosis) and inflammatory debris. The role of neutrophil reverse migration remains to be fully characterised in ARDS.
