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Review

# Understanding the roles of cytokines and neutrophil activity and neutrophil apoptosis in the protective versus deleterious inflammatory response in pneumonia

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SUMMARY

Inflammation is a double-edged sword in the outcome of pneumonia. On the one hand, an effective and timely inflammatory response is required to eliminate the invading respiratory pathogen. On the other, a toxic and prolonged inflammatory response may result in lung injury and poor outcomes, even in those receiving advanced medical care. This review focuses on recent understanding of the dynamics of the cytokine response, neutrophil activity, and responsiveness to cytokines and neutrophil lifespan as major elements of lung inflammation resulting in favorable or poor outcomes in lung infection primarily due to pneumococcus and influenza virus. Although some progress has been made in our understanding of the molecular mechanisms of the pneumonia inflammation axis composed of cytokines modulating neutrophil activation and neutrophil apoptosis, important questions remain to be answered. The degree of neutrophil activation, generation of reactive oxygen species, and the release of granule antimicrobial peptides play a key role in microbial pathogen clearance; however, prolonged neutrophil activation may contribute to lung injury and poor outcomes in pneumonia. Molecular markers of the mechanisms regulating neutrophil survival and apoptosis may help in the identification of novel therapeutic targets to modulate inflammation by inducing timely neutrophil apoptosis. A major task is to identify the mechanisms of dysregulation in inflammation leading to toxic responses, thereby targeting a biomarker and enabling timely therapies to modulate inflammation.



## 1. Introduction

### 1.1. The burden of pneumonia

Pneumonia accounts for a death toll greater than the combined mortalities due to malaria, tuberculosis, and AIDS.[1] A lack of appropriate management of the toxic inflammatory response remains a serious limitation in the medical management of bacterial and viral influenza pneumonia. The latter is illustrated by a mortality of about 50% in patients with severe pneumonia, despite receiving advanced medical treatment.[2] These results demand further research to better understand the mechanisms and factors regulating the host inflammatory response that lead to the poor outcomes associated with pneumonia.

### 1.2. The normal inflammation process in pneumonia

Pneumonia is an exuberant sequestration of peripheral neutrophils in the lungs, which is tightly regulated by cascades of cytokines produced by the immune system in response to an invading pathogen.[3] A network of cytokine signals plays an essential role in the modulation of the inflammatory response, the clearance of the pathogen, and the subsequent repair of the lung tissue. In pneumonia, as alveolar macrophages fail to control the invading pathogens, cytokines and chemokines are released in order to attract neutrophils to the affected lung

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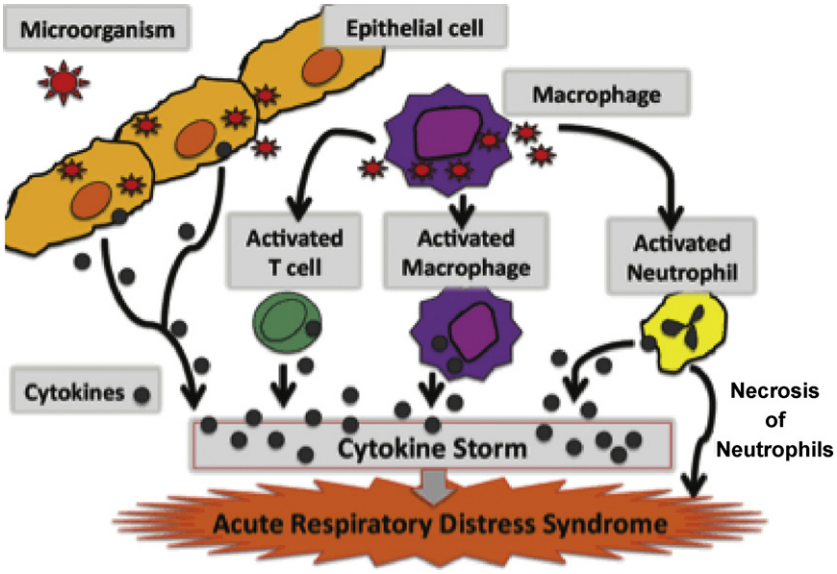


**Figure 1.** Proposed mechanism for the cytokine storm and poor clinical outcomes in community-acquired pneumonia (CAP) patients with deleterious inflammation.

area.[4–6] These mediators also induce resting neutrophils to transition to cells primed for enhanced responses and finally to fully activated cells. Activated neutrophils engulf and sequester microorganisms through phagocytosis and kill ingested bacteria by a combination of the production of toxic oxygen radicals, proteolytic enzymes, myeloperoxidase, defensins, and other bactericidal peptides. The degree of neutrophil activation, generation of reactive oxygen species (ROS), and the release of granule proteins play a key role in microbial pathogen clearance. In the ideal scenario, the acute lung inflammation is protective and self-limited, and once the infection has been controlled, cytokines also function to restore homeostasis, including the modulation of neutrophil apoptosis.[7] On the other hand, a cytokine storm results in a deleterious inflammation and poor clinical outcome (Figure 1).

### *1.3. Unmet needs in understanding the inflammation process in pneumonia*

The many acute lung inflammation-related factors responsible for lung injury and poor outcomes in pneumonia are not yet fully understood.[8] Data from the pre-antibiotic era indicate that the lung inflammatory response to *Streptococcus pneumoniae* is sufficient to protect a substantial number of patients.[9,10] This massive pulmonary inflammatory response has been considered 'beneficial', but it is now clear that in some patients it persists and evolves into a systemic toxic inflammatory response, leading to organ failure.[11,12] In severe pneumonia, such persistent and toxic inflammation leads to an overly pro-inflammatory cytokine balance, characterized by sustained high levels of tumor necrosis factor (TNF), neutrophil hyper-responsiveness, and dysregulation of lung neutrophil apoptosis, all major factors leading to lung injury and poor patient outcomes.[13,14] Consequently, efforts to improve the management of patients with pneumonia need to consider the host response factors, including the identification of biomarkers that might predict a poor patient outcome. In this regard, determination of the blood neutrophil phenotype in relation to cytokine profile, as well as assessment of blood neutrophil responsiveness and lung neutrophil apoptosis in relation to the degree of lung injury and clinical outcome should be instrumental in gaining an insight into both factors driving the toxic lung inflammation of community-acquired pneumonia (CAP) and potential therapeutic options.

From a translational research perspective, we reviewed studies of cytokines, neutrophil function, and neutrophil apoptosis modulating both the host inflammatory response and the outcomes of patients with CAP. A PubMed search was done using the phrases "cytokine and pneumonia", "neutrophil and pneumonia", and "neutrophil apoptosis and pneumonia" for the years 1980–2011. Other relevant papers on these topics dated earlier than 1980 were also included. This review examines the role of cytokines, neutrophil activity, and neutrophil apoptosis in the inflammatory response in pneumonia, with special emphasis on pneumococcal and influenza virus infections. We also describe some of our laboratory findings, relating inflammation to the clinical outcomes of patients with CAP.

## 2. Role of cytokines in the inflammatory response in pneumonia

### *2.1. Pro- and anti-inflammatory cytokines*

The exact role of each specific cytokine in the inflammatory response during pneumonia is still a subject of ongoing research. Although it is generally possible to classify cytokines into two groups based on their predominant function, i.e., pro-inflammatory cytokines (such as interleukin (IL)-1β, IL-1α, IL-6, IL-8 (CXCL8), IL-17, macrophage inflammatory proteins (MIP-1 (CCL3), MIP-1 (CCL4)), and TNF-α) and anti-inflammatory cytokines (such as IL-4, IL-10, and transforming growth factor (TGF)-β),[15–17] it is important to point out that this classification is not absolute, as many cytokines are capable of exerting both pro-inflammatory and anti-inflammatory effects depending on a variety of factors, such as the immunological and clinical contexts. During the initial phases of pneumonia, alveolar macrophages produce a variety of pro-inflammatory cytokines and chemokines whose role is to both attract and activate polymorphonuclear leukocytes necessary for local bacterial defense and clearance.[18,19] Thus, circulating levels of pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, IL-8, IL-12, and interferon gamma (IFN-γ), are usually found to be elevated in patients with CAP.[11,20,21] In addition to these 'classical pro-inflammatory cytokines', members of the IL-17/IL-23 axis have also been shown to play an important role in modulating airway inflammatory responses by regulating the expression and the pro-inflammatory versus tissue-protective properties of another cytokine, IL-22.[22,23]

Because of the potential to cause tissue damage, it is not surprising that inflammation is a tightly balanced process. In the context of infection, the pro-inflammatory cytokine response is normally regulated by the anti-inflammatory components of the immune system. However, if this balance cannot be achieved or becomes altered, deleterious effects can result for the whole organism.[24,25] Cytokines such as IL-10 and IL-4 play an anti-inflammatory role, inhibiting cytokine production and other pro-inflammatory functions by macrophages.[12] Indeed, IL-10 has been demonstrated as a key regulator of the degree of lung inflammation. For example, decreased IL-10 concentrations in bronchoalveolar lavage (BAL) fluid have been associated with a worse outcome in patients with acute respiratory distress syndrome (ARDS), presumably due to excessive inflammation.[26] Accordingly, it has been proposed that IL-10 could be a beneficial adjunctive therapy to antibiotics against pneumococcal pneumonia.[27]

Although most investigators tend to view cytokine responses in terms of pro- and anti-inflammatory balance, this is not the only interpretation. In contrast to the conventional interpretation of immunologic dissonance, defined as preponderance of either pro-inflammatory or anti-inflammatory cytokine responses, Kellum et al. have suggested that different patterns of cytokine response could best be described based on their concentrations as high, medium, and low, rather than their pro- or anti-inflammatory activities.[28]

### 2.2. Cytokine production: the role of the bug

Although there is no clear relationship between the local bacterial burden and the intensity of the elicited inflammation, it has been suggested that the host immune response to CAP and the magnitude of cytokine secretion vary depending on the causative microorganism involved.[12,21,22] A recent review from Paterson and Orihuela showed that a variety of pro-inflammatory cytokines (IL-6, IL-12, IL-17, and IL-18) are important mediators in the innate response to pneumococci.[29] Their concentrations in serum are usually higher in pneumococcal pneumonia than in non-pneumococcal lung infection.[30] In addition, TNF-α has also been reported to be a critical factor in the protective response against pneumococci.[31] These findings are supported by experimental evidence that deletion or neutralization of TNF-α has deleterious effects and by the finding that human patients treated with anti-TNF-α therapies suffer an increased risk of invasive pneumococcal disease.[30] It is important to remark that while a pro-inflammatory cytokine response is essential in the response to pneumococcal pneumonia, the same cytokines can cause deleterious effects if the response becomes excessive or not adequately balanced by anti-inflammatory mechanisms.

Pro-inflammatory cytokine responses are also essential during immune responses to influenza pneumonia, but an overly aggressive and dysregulated cytokine response, known as a 'cytokine storm', has been associated with influenza-related morbidity and mortality, particularly in avian H5N1 influenza.[32] Several studies have recently been conducted in order to better understand the inflammatory response during influenza H1N1 virus infection. Patients with influenza pneumonia had significantly higher serum levels of cytokines than those without a lung infection,[33] presumably due to the hyper-reactivity of immune cells in response to acute lung injury.[34] Consistent with the general findings in CAP, disease severity was associated with pronounced impairment of the host immune response.[35] Furthermore, apart from tissue damage caused by the virus lytic replication, lung damage can be worsened by an imbalanced overproduction of antiviral cytokines. In this regard, Pinto et al. demonstrated that virus titers and pro-inflammatory cytokine expression can be modulated simultaneously, providing a new potential therapy against viral pneumonia.[36]

In an elegant paper, Menéndez and co-workers showed that different inflammatory patterns are elicited by microorganisms and that this finding may provide a useful tool for both diagnosis and treatment.[37] Finally, understanding the different cytokine responses elicited by different pathogens is crucial because, as was suggested by McConnell et al., targeting specific host inflammatory responses (induced by each pathogen) could represent a new potential therapeutic approach in sepsis.[38]

### 2.3. Cytokine timing and decompartmentalization

In addition to antibiotic therapy, the inflammatory response, over time, is crucial to control the infection and eradicate the pathogen in pneumonia. Mean cytokine concentrations are generally highest on admission and they decline rapidly over the first few days.[24,28] It has been proven that IL-1, IL-6, IL-8, and IL-10 act as acute-phase proteins, decreasing rapidly after admission, in contrast to the more blunt kinetics of C-reactive protein.[30] However, the circulating cytokine response during pneumonia remains a heterogeneous process and continues for more than a week after presentation, with considerable overlap between those who do and those who do not develop severe sepsis.[28] In an ongoing study by our group comparing cytokine profiles in patients differing in 'time to clinical stability' (TCS) following hospital admission, we found that although the levels of pro-inflammatory (IL-6, IL-8, IL-12) and anti-inflammatory (IL-10) cytokines were generally high on admission (day 1) for all patients, there was a sharp decrease in those with an early TCS (>3 days), but a plateau in those with a late TCS (>3 days).

In pneumonia the host response is generally compartmentalized, with markedly different concentrations of inflammatory mediators – cytokines included – in the lungs versus the systemic circulation.[38] TNF-α and IL-6 act as general markers of inflammation in pneumonia and regulate the expression of acute-phase proteins, thus integrating systemic response and local injury.[19,39–41] Despite this compartmentalization, Calbo et al. demonstrated that in patients with severe pneumococcal pneumonia, pro- and anti-inflammatory responses could be detected in venous blood, representing a systemic extension of the compartmentalized response, where each cytokine showed a similar pattern of decline, with progressive normalization accompanied by clinical recovery, suggesting that this phenomenon is a part of the homeostatic response to the infection.[20] Moret et al. have recently hypothesized that inflammatory resolution can proceed at different rates, being slower in lung compared to the systemic compartment.[42] In an ongoing study comparing cytokine levels in plasma vs. sputum samples from pneumonia patients, our group has observed that although the levels of most cytokines are higher in the latter, different cytokines can be grouped based on their relative concentration ratios between the two types of samples (e.g., sputum/plasma). For example, these ratios were >1000-fold higher for IL-1b, IL-1ra, and IL-8, whereas <1 for IL-10 and IL-12 in sputum vs. plasma (see Table 1). These results suggest that there is not only true compartmentalization but preferential cytokine secretion locally vs. systemically.

**Table 1**
Cytokine concentration ratios in sputum vs. plasma[a]

| Sputum/plasma ratio | | | |
|---|---|---|---|
| >1000 | 100–200 | 1–20 | <1 |
| IL-1b | IFN-γ | IP-10 | IL-10 |
| IL-1ra | IL-6 | IL-17 | IL-12p70 |
| IL-8 | TNF-α | | |

IL, interleukin; IFN-γ, interferon-gamma; IP-10 interferon gamma-induced protein 10; TNF-α, tumor necrosis factor alpha.

[a] Ratios in the table were calculated by dividing the concentration in sputum (in pg/ml) by the concentration in plasma (in pg/ml) for each cytokine.

### 2.4. Cytokines and clinical outcomes

There is increasing evidence that an adequate cytokine balance plays a crucial role in determining outcomes in hospitalized patients with pneumonia.[25] Different markers of severity of the disease such as confusion, hypotension, pleural effusion, and bacteremia have been associated with excesses of both pro- and anti-inflammatory cytokines, particularly IL-6 and IL-10.[22,24,43,44] Serum IL-6 correlates best with both disease-specific and generic severity scores and may therefore be a valid marker of inflammation and prognosis in CAP.[21,24] A systemic inflammation characterized by an increase in IL-6 and IL-10 has also been correlated with delayed clinical stability, while pre-antibiotic treatment as well as pneumococcal vaccination seem to be protective factors for excess of IL-6 and IL-10.[42,43]

The evaluation of circulating cytokine concentrations could help in predicting the risk of potential complications in CAP. A persistent, up-regulated, pro-inflammatory response is associated with deaths due to cardiovascular disease, renal failure, infection, and cancer, whereas persistent low-grade inflammation and immune suppression may play an important role in coronary events, cerebrovascular events, and repeat bouts of CAP.[45–49] Patients who have persistent elevation of cytokines have a higher risk of dying, and mortality seems to be correlated with the ratio of IL-6 to IL-10.[22,43,50,51] However, some authors suggest that even if plasma levels of IL-6 strongly predict outcomes, they are probably not actually correlated to mortality.[51,52] Cytokine levels might actually be more predictive of adverse outcomes when measured at the end of hospitalization, rather than on admission.[28] This hypothesis is supported by the strong association between elevated circulating IL-6 concentrations and higher mortality over the subsequent 3 months after discharge.[45] In the same way, serum TNF-α levels can be useful in identifying patients at risk of functional impairment following hospitalization with CAP.[53]

A recent study was conducted by our group based on the hypothesis that gradients of pro- and anti-inflammatory cytokines in blood and in exhaled breath condensate correlate with the degree of lung injury and clinical outcome of patients with CAP.[54,55] A negative correlation was found between the IL-10/IL-6 ratio and TCS ($r = -0.372$, $p = 0.014$), as well as IL-4/IL-6 ratio and TCS ($r = -0.312$, $p = 0.042$). These findings may be due to the balancing role of anti-inflammatory cytokines. An effective anti-inflammatory response seems to be a protective factor, whilst individuals showing unbalanced pro-inflammatory patterns take a longer time to recover. Cytokine production may also vary according to the individual's characteristics including age, smoking status, and co-existing medical conditions. Kelly et al. found that advanced age was not associated with blunting of the systemic cytokine response, although in well-functioning, elderly subjects, pre-infection systemic levels of TNF-α and IL-6 were associated with a higher risk of CAP requiring hospitalization in smokers and in those with coexisting medical conditions.[56] Inflammatory responses in immunocompromised patients are still not completely understood. In *Pneumocystis jiroveci* pneumonia, the concentrations of monocyte chemotactic protein-1/2 (MPC-1/2) (CCL2/CCL8), IL-8, and IL-6 differed according to the underlying disease, tending to be higher in patients with autoimmune disease and lower in those with AIDS.[57] Moreover, it was recently demonstrated that local TNF-α production is reduced in neutropenic patients, which may be a significant factor determining the severity of pneumonia and a possible reason for the poor prognosis in these patients.[58]

### 2.5. Modulation of pro-inflammatory cytokine production and clinical outcomes

Because of the key importance of the inflammatory response in the outcome of pneumonia, anti-inflammatory therapies have been considered to have potential beneficial effects. Based on their well-established inhibitory effects on the production of a variety of pro-inflammatory cytokines such as TNF-α, IL-1, IL-2, IL-6, and IL-8, the use of glucocorticoids has been promoted as adjunctive therapy in severe pneumonia. The effectiveness of glucocorticoid treatment in pneumonia, however, has remained somewhat controversial, in part because of its added immunosuppressive and other effects, particularly after prolonged administration. Some reports seem to support a sort of modulation of the inflammatory response in severe CAP by short-term administration of glucocorticoids, with a favorable impact in terms of accelerating the time to resolution of symptoms, length of stay in the intensive care unit, and mortality.[59,60] On the other side, a different experience suggested no evidence of a beneficial effect of corticosteroids in patients with ARDS secondary to influenza pneumonia.[61] Additional studies are needed to more completely characterize and optimize the therapeutic potential of glucocorticoids in pneumonia.

## 3. Role of neutrophils in the inflammatory response in pneumonia

### 3.1. Neutrophil strategies to combat the infection

Neutrophils have evolved different strategies and mechanisms to combat an infection. These cells have the ability to kill microorganisms both by the production of ROS and by the release of antibacterial and lytic enzymes inside the phagosome.[62] Additionally, neutrophils can release their genomic DNA and, in combination with elastase and other antimicrobial granule proteins, generate a net called a DNA-based neutrophil extracellular trap (NET) that will trap and kill microbial pathogens.[63] Besides the ability of neutrophils to kill microbial pathogens, these cells can release granule components and a variety of cytokines and chemokines resulting in the recruitment and activation of other cells of the immune system, such as monocytes, dendritic cells, and T-cells, which provide evidence for the key role that neutrophils play in the regulation of the host immune response.[64–67]

Neutrophils have four different types of granules: secretory vesicles, gelatinase, specific, and azurophilic granules, which are differentiated based on their protein content and separation by a density gradient fractionation.[67,68] The release of the different neutrophil granules is sequential during the inflammatory response. The secretory vesicles are the first granules to undergo exocytosis and they allow neutrophils to adhere and roll through the endothelial monolayer in the blood vessels. Release of gelatinase granules will follow, allowing neutrophils to transmigrate into the site of infection. Once the neutrophils arrive at the site of inflammation, specific and azurophilic granule proteins come out to support bacterial killing by the release of antimicrobial proteins.[69–71]

### 3.2. Neutrophil activation and migration

After initiation of a bacterial infection, a number of chemokines, cytokines, and chemoattractants are released by surrounding cells, which activate neutrophils that are present in the circulation during rest such that their harmful intracellular granule contents are not accidentally released to damage host tissue.[67] In patients with pneumonia, one of the primary defense mechanisms is the migration of neutrophils into the alveolar space. In the course of this migration process, neutrophils change their phenotype from a resting circulating cell into a prime cell. Additionally, resting neutrophils can be primed by bacterial products: cytokines and chemokines including IL-8, IFN-γ, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and platelet activating factor

(PAF).[72] Primed neutrophils have the ability to trigger a 10–20-fold increase in the respiratory burst response when they encounter a second stimulus at the site of inflammation, such as the bacterial wall component N-formyl-methionyl-leucyl-phenylalanine (fMLF).[73]

Activation of neutrophils by priming agents is an advantageous process to clear infection, but uncontrolled ROS production and release of bactericidal proteins from the neutrophil granules can result in organ damage. We have recently shown that exocytosis of neutrophil granules plays a critical role in TNF-α-induced priming. Blocking secretory vesicle, specific granule, and gelatinase granule exocytosis by introduction of a TAT-SNAP-23 fusion protein reduced phagocytosis-stimulated hydrogen peroxide production without affecting phagocytosis or bacterial killing inside the phagosome.[74]

It has been reported that patients with severe sepsis have a primed population of neutrophils.[14] Priming of ROS production is involved in many human diseases. Ex vivo stimulation of neutrophils from ARDS patients showed that those cells were hyper-responsive with the ability to produce high levels of ROS. Additionally, it was shown that the hyper-responsiveness of neutrophils derived from ARDS patients correlated with high plasma levels of TNF-α. In summary, TNF-α-primed neutrophils may play a major role in the pathogenesis of ARDS-associated lung injury.[13] Understanding the mechanisms that regulate neutrophil granule exocytosis in primed cells during infection can provide new insights into potential therapeutic options to control neutrophil activation during inflammation.

We are currently conducting a translational study to evaluate the degree of the neutrophil functional response over time in the blood and the lungs of hospitalized patients with CAP. Blood and sputum samples from CAP patients have been collected at different time points after hospitalization. Different neutrophil function assays such as exocytosis of the four different granule subsets, phagocytosis, respiratory burst activity, and apoptosis have been evaluated in both blood and sputum samples. Basal levels for each of the neutrophil functional assays have been determined using whole blood from healthy donors. Our preliminary results indicate that a prolonged, primed neutrophil phenotype in the circulation, accompanied by low plasma levels of IL-6 and IL-1β, result in a delay to clinical stability in patients with CAP (S.M. Uriarte et al., manuscript in preparation).

## 4. Role of neutrophil apoptosis in the inflammatory response in pneumonia

Neutrophils are terminally differentiated cells and have a very short lifespan. They are viable in the circulation for 8–10 h before they succumb to cell death. It is very important to maintain the cellular homeostasis of neutrophils. It has been shown that under physiological conditions the turnover rate for neutrophils is equivalent to the production of these cells from the bone marrow, which will constitute the circulating neutrophil population, and the rate of neutrophils utilized in the tissue.[75] Constitutive neutrophil death occurs in the absence of extracellular stimuli and this is the essential mechanism to maintain neutrophil homeostasis. Increased rates of neutrophil death can cause neutropenia by decreasing neutrophil counts. This can augment the chances of contracting bacterial and fungal infections. Moreover, delayed neutrophil death causes neutrophilia by increasing neutrophil counts; this is associated with increased inflammation after infection. Thus, maintaining appropriate neutrophil numbers or neutrophil homeostasis is critical during infection and/or inflammation. During infection or inflammation, the neutrophil lifespan is prolonged or neutrophil death is delayed to combat infection and inflammation.[76] However, delayed neutrophil apoptosis can cause tissue damage by generation of ROS. Delayed neutrophil death can be modulated by induction of apoptosis or necrosis. It has been shown that apoptotic or necrotic neutrophils release α-defensins, which have anti-inflammatory properties on macrophages by inhibiting the release of pro-inflammatory cytokines without affecting their antimicrobial activity.[77] Thus, mechanisms that lead to timely induction of apoptosis are critical in resolving the inflammatory response.[7]

Once neutrophils undergo apoptosis, they express phosphatidylserines on the outer leaflet of the cells so that they are recognized and cleared by scavenger macrophages.[78] The ideal scenario for resolution of inflammation is the termination of neutrophil activation by induction of neutrophil apoptosis after they phagocytose dead bacteria. Neutrophil apoptosis occurs without release of harmful granule contents. However, during infection or inflammation the neutrophil lifespan is prolonged and the continued generation of superoxide radicals by these activated neutrophils may cause tissue damage to surrounding normal tissue. When phagocytosis is impaired, neutrophils undergo necrosis. Necrotic cell death is accompanied by release of harmful granule contents that further damage healthy tissue and exacerbate the inflammatory response by recruiting additional inflammatory cells into the site of infection. Thus, uncontrolled neutrophil activation, by delay of neutrophil apoptosis and inadequate resolution of inflammation, plays a critical role in the tissue injury associated with septic multi-organ failure syndrome, ischemia–reperfusion injury observed in myocardial infarction, stroke, and acute tubular necrosis, as well as immune complex-mediated diseases.[79–84]

For complete resolution of inflammation to occur, apoptotic neutrophils have to be cleared by macrophages thereby preventing release of toxic neutrophil granule proteins and preserving the host tissue. Clearance of apoptotic neutrophils can occur both at the systemic level and at the tissue level. Circulating neutrophils will be cleared in the liver, spleen, and bone marrow, while apoptotic tissue neutrophils will be cleared by local tissue macrophages. However, if either neutrophil apoptosis or clearance of apoptotic neutrophils by macrophages is delayed, inflammation-related tissue damage occurs. In some scenarios, neutrophil clearance is not delayed, likely because of the presence of specific cytokines and/or a pro-inflammatory milieu resulting in an exacerbation of inflammation by macrophages presenting neutrophils to the T-cells as non-self. Neutrophil apoptosis may also not be delayed but dysregulated or compensated by the neutrophil NETosis or autophagy modulating neutrophil proteins, leading to presentation of these neutrophil-modulated proteins by antigen presenting cells to T-cells as non-self antigens, leading to stimulation of the immune system and exacerbation of inflammation. It is therefore important to understand the mechanisms that regulate neutrophil apoptosis. This phenomenon seems straightforward, but if either neutrophil apoptosis is delayed or the clearance of apoptotic neutrophils by macrophages is delayed, this leads to exacerbation of inflammation.

There are two distinct pathways that contribute to apoptosis: intrinsic and extrinsic apoptotic pathways. The extrinsic pathway is the death receptor pathway activated by Fas (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL).[85] Ligation of the death receptors at the cell surface leads to the formation of death-inducing signaling complex (DISC), involving adaptor proteins such as Fas-associated death domain (FADD) and activation of caspase 8.[86] The intrinsic pathway is a mitochondrial pathway stimulated by cell stressors such as UV radiation, heat shock, growth factor withdrawal, osmotic shock, DNA damage, and chemotherapeutic drugs. The intrinsic pathway is mediated

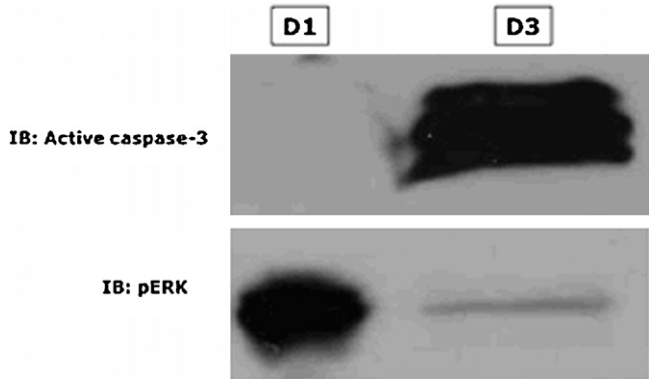


**Figure 2.** Increased ERK phosphorylation (IB: pERK) and no caspase 3 activation in neutrophils obtained from a patient sputum on day 1 (D1), indicative of increased neutrophil survival and activation. Sputum neutrophils from the same patient on day 3 (D3) demonstrate increased caspase 3 activation and abrogation of ERK phosphorylation, indicative of termination of neutrophil activation and induction of neutrophil apoptosis.

by generation of ROS leading to a decrease in mitochondrial transmembrane potential, release in cytochrome C and apoptosis-inducing factor, assembly of the apoptosome, and finally, the activation of caspase 3.[87,88] Both the extrinsic and intrinsic pathways culminate in activation of caspases, which are known to cleave intracellular proteins resulting in the death of the cell.

Neutrophil apoptosis is tightly regulated by a complex network of signaling pathways that control activation and inactivation of Bcl2 family proteins, including anti-apoptotic proteins A1 and Bcl-xL, Mcl-1, and pro-apoptotic proteins Bad, Bid, Bak, and Bax. Removal of activated neutrophils by apoptosis is a significant mechanism by which the inflammatory response is appropriately terminated.

Extensive work has been done on the involvement of several kinases that regulate neutrophil survival. Pro-survival kinases MAPK/ERK and PI-3K/Akt have been shown to regulate neutrophil survival.[87] Thus, ERK phosphorylation is a marker of neutrophil activation or survival and active caspase 3 is a marker of neutrophil apoptosis.

Pneumonia is also associated with neutrophil activation in the sputum and BAL fluid. Spontaneous apoptosis of sputum neutrophils in pneumonia patients is markedly reduced in non-responding CAP, while it is increased in responding CAP patients, allowing resolution of inflammation in the responding group.[42] The Community-Acquired Pneumonia Inflammatory Study Group (CAPISG) evaluated neutrophil activation in the sputum of CAP patients. The inflammatory response was correlated with clinical outcomes in CAPISG patients. In one patient who was confirmed to have influenza A H3N2 pneumonia by nucleic acid amplification tests for respiratory viruses, on day 1 (D1) of admission, we demonstrated increased ERK phosphorylation and no caspase 3 activation in neutrophils obtained from the sputum of this patient (Figure 2). The data are indicative of increased neutrophil survival and activation. On day 3 (D3), sputum neutrophils obtained from the same patient demonstrated increased caspase 3 activation and abrogation of ERK phosphorylation, indicative of termination of neutrophil activation and induction of neutrophil apoptosis (Figure 2). Therefore, it is critical to identify signaling proteins within the complex network of proteins that cross-talk with one another to either promote neutrophil survival or neutrophil apoptosis. The identification of such proteins may lead to the discovery of novel therapeutic targets to control inflammation by inducing timely neutrophil apoptosis.

## 5. Conclusions

There is substantial growing knowledge of the causal effect of the toxic inflammatory response in the poor outcomes of severe pneumonia managed with appropriate medical care. A prolonged toxic inflammatory response is primarily driven by a dysregulation of cytokine cascades, neutrophil hyperactivity, and increased neutrophil survival, resulting in lung injury. The degree of neutrophil activation, generation of ROS, and the release of granule proteins, all play a key role in microbial pathogen clearance; however, prolonged neutrophil activation may contribute to lung injury and poor patient outcomes. Molecular markers of regulation of neutrophil survival and neutrophil apoptosis may lead to the discovery of novel therapeutic targets to modulate inflammation by inducing timely neutrophil apoptosis. A major task is to identify early cytokine dysregulation leading to a toxic inflammatory response, thereby targeting a biomarker and enabling timely therapies to modulate inflammation.

*Conflict of interest:* No conflict of interest to declare.

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