Pulmonary immune cells in health and disease: Polymorphonuclear neutrophils

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ABSTRACT: The pulmonary vasculature represents the largest reservoir of polymorphonuclear neutrophils (PMNs) in the human body. This is striking contrast with the paucity of PMNs present in the normal airways and alveoli.

However, the respiratory tract constitutes an easy access for microorganisms and particles present in inhaled air and, therefore, efficacious defence mechanisms are required. When the mucociliary clearance and the alveolar macrophages are overwhelmed, the rapid recruitment of PMNs from the lung vasculature appears to be a crucial response of the host against the pathogens. The regulation of adherence of PMNs to endothelial cells (EC), followed by the transendothelial migration are now better understood, and are under the control of a series of adhesion molecules modulated by bacterial and inflammatory mediators.

In addition to their defensive role, PMNs have also been implicated in acute and chronic injurious diseases of the lung. Clearly, PMNs contain enough cytotoxic and proteolytic material to induce lesion changes. However, the release of this material is likely to be dependent on environmental factors, including mediators derived from other inflammatory and immune cells. The presence or absence of these factors could explain the fact that high numbers of PMNs can be observed in the airways and alveoli without major lesions whilst in other conditions, a marginal increase of PMNs in the respiratory tract can be associated with major damage and irreversible architectural changes in the lung.

In the absence of an inflammatory process, the polymorphonuclear neutrophils (PMNs) are almost exclusively confined to the intravascular compartment, where they comprise the majority of circulating leucocytes. However, in response to inflammatory stimuli, a rapid and often massive influx of PMNs can be driven to the inflamed tissues. This is particularly true in the lung, where PMN traffic can be facilitated both by a rather thin barrier separating the alveolar space from the capillary lumen and by the sequestration of PMNs in the pulmonary vasculature. Some of these PMNs appear to adhere to the endothelium of the postcapillary venules, even in normal conditions, providing a pool of rapidly recruited cells.

The PMNs are considered as major effector cells in acute inflammatory processes, and their role in the defence of the host against bacterial and fungal invasion is well-documented. This role is best illustrated by the frequent and severe infections associated with neutropenia. More recently, PMNs have also been implicated in the pathogenesis of tissue injury related to chronic inflammation, generally leading to lung fibrosis. The purpose of the present review is to discuss the function of PMNs, and their migratory and secretory profile in the normal lung, as well as in pulmonary diseases.

Origin, maturation and ageing

PMNs have an estimated life-span of 6–8 h in peripheral blood and this probably explains their production at the astonishing rate of 2.5 billion cells·h⁻¹ [1]. Like other leucocytes, PMNs originate from a common pluripotent stem cell in the bone marrow. In order for the bone marrow to follow the PMN turn-over, two conditions are required: the maintenance of enough stem cells and the differentiation of these cells towards mature cells, under the control of several growth factors [2–4]. The totipotent stem cells are the progenitors for all blood cells, including lymphocytes, and can be recognized by their ability to form blast colonies in vitro. The totipotent stem cell is the precursor of the colony forming units (CFU) - multipotent cell (GEMoMe). The GEMoMe, characterized by a limited ability of self-renewal, represents the common source for granulocytes (G), erythrocytes (E), monocytes (Mo) and megakaryocytes (Me). The GEMoMe differentiates to the CFU - granulocyte-monocyte (G/Me), and then to the CFU - granulocyte cells, the precursors of PMNs. In contrast to PMNs and monocytes, the bone marrow stem cells of eosinophils and basophils are still the subject of controversy.

Within the last decade, a series of growth factors for
bone marrow stem cells have been identified and their gene cloned. Briefly, three groups of glycoproteins have been characterized according to their effects on progenitor cells. The first group of molecules act preferentially on early progenitor cells and, unlike true growth factors, they stimulate cell differentiation and act synergistically with other factors. This group includes interleukin (IL)-1, IL-4 and IL-6. A second group, essentially IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF), appear as the major growth factors, since they induce multipotent stem cells (GEMoMe) and progenitor cells of the myeloid, erythrocyte and megakaryocyte lineage to form colonies. The last group of growth factors which include G-CSF for PMNs regulates the differentiation of more committed stem cells towards mature granulocytes. When leaving the bone marrow, the PMNs are fully differentiated and equipped with the complete spectrum of surface receptors and intracytoplasmic granules with their secretory products. For instance, the transcription of messenger ribonucleic acid (mRNA) for neutrophil elastase and myeloperoxidase is only observed in myelocytic precursor cells and not in mature PMNs [5].

In addition to their role on bone marrow progenitor cells, growth factors also act on mature leucocytes. Thus, GM-CSF can modulate PMN migration and degranulation, via the binding of specific surface receptors (see below) [6, 7].

The large scale production of recombinant forms of these growth factors (M-, G-, GM-CSF and IL-3) has enabled their use in clinical trials to accelerate bone marrow recovery, particularly after chemotherapy. Randomized studies are under way to evaluate the benefit provided by such adjuvant treatments [2].

Whilst the production of granulocytes is critical for the host, the disposal of these cells appears to be equally crucial. In particular, with the impressive number of PMNs continuously produced by the bone marrow, it is important to consider the fate of these phagocytes in the blood and in tissues. Among the mechanisms implicated in the removal of PMNs, apoptosis or programmed cell death appears to play a central role. Apoptosis is characterized by morphological changes, including chromatin condensation and cytoplasmic vacuolization [8]. This process is associated with deoxyribonucleic acid (DNA) fragmentation, and can be induced by ageing PMNs in culture for 24 h. Recently, the aged PMNs were found to be specifically recognized and phagocytosed intact by alveolar macrophages, suggesting one mechanism of disposal of senescent PMNs from the respiratory tract, thereby preventing further release of potentially toxic secretory products [9].

Membrane receptors and membrane associated proteins

A series of membrane receptors have been identified on the surface of PMNs and are listed in table 1. These receptors constitute the link between PMNs and their environment, and modulate PMN functions, including adherence, migration, degranulation and phagocytosis.

### Table 1. - List of polymorphonuclear neutrophil (PMN) major surface ligands

<table>
<thead>
<tr>
<th>Surface ligand</th>
<th>[Ref.]</th>
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<tbody>
<tr>
<td><strong>Complement component</strong></td>
<td></td>
</tr>
<tr>
<td>CRI</td>
<td>[10]</td>
</tr>
<tr>
<td>C3b (CR3)</td>
<td></td>
</tr>
<tr>
<td>C5a R</td>
<td>[11]</td>
</tr>
<tr>
<td>Decay accelerating factor (DAF)</td>
<td>[12]</td>
</tr>
<tr>
<td>Membrane cofactor protein</td>
<td>[12]</td>
</tr>
<tr>
<td><strong>Immunoglobulins</strong></td>
<td></td>
</tr>
<tr>
<td>FcyRII and FcyRIIB</td>
<td>[13, 14]</td>
</tr>
<tr>
<td>FcεR</td>
<td>[15]</td>
</tr>
<tr>
<td>IgE (S-lectin, Mac2/εBP)</td>
<td>[16]</td>
</tr>
<tr>
<td><strong>Bacterial peptides and endotoxins (LPS)</strong></td>
<td></td>
</tr>
<tr>
<td>FMLP (PPR)</td>
<td>[17]</td>
</tr>
<tr>
<td>LPS (CD14 and BIP)</td>
<td>[18, 19]</td>
</tr>
<tr>
<td><strong>Colony stimulating factors</strong></td>
<td></td>
</tr>
<tr>
<td>G and GM-CSF</td>
<td>[20]</td>
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<tr>
<td><strong>Chemotaxins</strong></td>
<td></td>
</tr>
<tr>
<td>NAP-1/IL-8, NAP-2, gro/MGSA</td>
<td>[21]</td>
</tr>
<tr>
<td>LTβ</td>
<td>[22]</td>
</tr>
<tr>
<td>PAF</td>
<td>[23]</td>
</tr>
<tr>
<td><strong>Adhesion molecules</strong></td>
<td></td>
</tr>
<tr>
<td>β1-integrins (LFA-1, Mac-1, p 150,95)</td>
<td>[24]</td>
</tr>
<tr>
<td>L-selectin</td>
<td>[24]</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>ANP</td>
<td>[25]</td>
</tr>
<tr>
<td>Adenosin (A1)</td>
<td>[26]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>[27]</td>
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</table>

IgE: immunoglobulin E; LPS: lipopolysaccharide; FMLP: formyl methionyl-leucyl-phenylanine; PPR: formyl peptide receptor; BIP: bacterial permeability increasing protein; G and GM-CSF: granulocyte and granulocyte macrophage colony-stimulating factor; NAP: neutrophil-activating peptide; IL-2: interleukin; MGSA: melanoma growth stimulatory activity; LTβ: leukotriene B2; PAF: platelet-activating factor; LFA-1: lymphocyte function antigen-1; ANP: atrial natriuretic peptide; TNF-α: tumour-necrosis factor-α.

Recent studies have focused both on molecular and on functional characterization of PMN surface molecules. It has been known for many years that immunoglobulin (Ig) G can mediate phagocytosis, both in PMNs and in macrophages [28, 29]. Also, IgG immune complexes are potent inducers of PMN migration and degranulation, and have, therefore, been implicated in the recruitment and activation of PMNs in inflammatory processes [30].

Two receptors for the complement activating fragment (Fc) portion of IgG (FcεRI) have been characterized on PMNs: FcεRII and FcεRIIB, two low affinity receptors binding preferentially aggregated forms of IgG [13, 14]. Receptors for IgA (FcεR) have also been identified on the cell surface of PMNs. This receptor is a 60 Kd protein, and appears structurally similar to the FcεR described on mononuclear phagocytes [15]. The function of FcεR in PMNs is still the subject of controversy, in particular its role in PMN mobility. Thus, some authors have found an inhibition of PMN migration towards an IgA concentration gradient (chemotaxis), whilst others have reported an increase in PMN random migration (chemokinesis) in the presence of IgA [31, 32]. Moreover,
IgA can mediate phagocytosis in PMNs, and haematopoietic growth factors, such as GM-CSF and G-CSF can enhance this function, via an increase in FceRI expression on PMNs [33]. This suggests a synergistic mechanism between IgA and growth factors for PMN activation, since a limited number of high affinity receptors for G and GM-CSF have been demonstrated on mature PMNs [20].

Two receptors for IgE have been described: FceRI, a high affinity receptor present on mast cells, and FceRII, a low affinity receptor present on macrophages, and recently found to be similar to FcyRII and RIII, at least in mice [34, 35]. None of these receptors are present on PMNs. However, PMNs can interact with IgE through its binding to the Mac-2/ε-bonding protein of the S-lectin family present on their surface [16]. This interaction appears to account for the activation of the PMN respiratory burst by IgE immune complexes.

Bacteria-derived peptidolipids and their synthetic analogues formyl-methionyl-leucylphenylalanine (fMLP) are potent activators of PMN chemotaxis and respiratory burst. These peptides interact with PMN via a specific formyl peptide receptor (FPR) [17]. The intracellular signal triggered by FPR activation involves a cytosolic protein. Several molecules including G and GM-CSF can upregulate the cell surface expression of FPR and, recently, FceRIIIB was shown to modulate the FPR-mediated functions via a mechanism which probably involves an intracellular transduction pathway common to the two receptors [14]. Thus, binding of aggregated IgE to the FceRIIIB diminishes the subsequent chemotactic response of PMN to fMLP. This effect appears to be selective, since PMN response to other chemoattractants is not altered. Endotoxin (lipopolysaccharide (LPS)), derived from Gram-negative bacteria, also interacts with the PMN surface via the binding of several ligands. Among these ligands, the LPS-binding protein (CD14) and the bacterial permeability increasing protein (BIP) have been reported to be the predominant binding sites for LPS on PMNs [18, 19].

Most of the PMN chemoattractants also act on PMNs via binding to specific membrane receptors. This is now established for neutrophil activating peptide (NAP)-I/IL-8 which shares two classes of PMN receptors with two other PMN activators, namely NAP-2 a closely related variant and gro/melanoma growth-stimulatory activity (gro/MGSA) [21]. Also, the arachidonic acid metabolite leukotriene B₄ (LTB₄) binds to specific receptors present on PMNs [22].

Receptors for complement fragments represent an important group of surface ligands on PMNs. Several receptors for the complement component C3 have been reported in PMNs [10]. Thus, CR1 is primarily implicated in immune complexes binding and processing, while CR3, the iC3b receptor also called Mac-1 or MoI and classified as CD11b/C18, is part of the CD18 complex (β₂ integrins) together with lymphocyte function antigen-1 (LFA-1, CD11a) and p 150,95 (CD11c) [24]. The CR3 is primarily involved in phagocytosis, but also plays an important role in adherence of PMNs to endothelial cells (EC). Two additional C3-binding proteins, namely the decay accelerating factor (DAF) and the membrane cofactor protein (MCP) are present on PMNs, and essentially protect tissues and cells carrying these binding proteins from unrestrained C3 and C5 convertase activation [12]. CR1, DAF and MCP, together with CR2 present on B-cells, some T-lymphocytes and epithelial cells, form the regulators of complement activation (RCA). C5a receptors are also present on PMNs and mediate adherence, chemotaxis, degranulation and oxygen radical release induced by C5a [11]. In contrast, with CR1 and CR3, C5a receptors are not upregulated by secretogogues [36].

Finally, PMNs express, on their cell membrane receptors for adenosine, tumour necrosis factor-alpha (TNF-α) and atrial natriuretic peptide (ANP) [25–27]. Although, as single agents the latter two have minimal effect on PMNs, they can potentiate PMN activation induced by other factors, such as fMLP. Adenosine also enhances fMLP-induced chemotaxis, and this activity was found to be mediated by the engagement of adenosine 2 (A₂) receptors present on PMNs.

**Adherence and migration**

A rapid extravasation of PMNs towards infected tissues is crucial for the defence of the host against invading microorganisms. In the lung, two thirds of intravascular PMNs adhere loosely to the endothelium, forming the marginated pool of PMNs. Recent studies have characterized a series of molecules involved in adherence between PMNs and ECs [37]. The adhesion molecules on PMNs include β₂-integrins (LFA-1, Mac-1 and p 150,95) and L-selectins. These PMNs adhesins bind to corresponding molecules present on the surface of ECs, mostly in postcapillary venules. The regulation of expression of adhesion molecules, both on PMNs (or monocytes) and ECs is critical for adherence and migration of leukocytes. In particular, the importance of integrins is well illustrated by the frequent episodes of infection in patients with β₂-integrins deficiency. These patients are neutropenic, and their PMNs display impaired adherence and chemotaxis.

Compared to PMNs, the role of ECs in the regulation of migratory processes is likely to be equally important, as documented by recent literature. Thus, binding properties have now been demonstrated for a series of EC surface proteins, including P-selectin and the intercellular adhesion molecule-1 and 2 (ICAM-1/ICAM-2), members of the Ig supergene family [38]. ICAM-1 is expressed on unstimulated ECs and represents the corresponding binding site for both LFA-1 and Mac-1 of the PMNs, whilst ICAM-2 only binds LFA-1. Binding of ICAM-1 to β₂-integrins is involved in adherence, and appears to play a major role in transendothelial migration of PMNs.

Several mechanisms can increase PMN adherence to ECs. Firstly, β₂-integrins and ICAM-1 expression on the cell surface can be upregulated, providing an increased number of anchorage sites. This upregulation can be induced by cytokines, such as TNF-α and IL-1 for ECs and by GM-CSF and bacteria-derived formyl peptides
for PMNs [36]. Secondly, in the absence of upregulation of adhesion molecules, the interaction between ECs and PMN ligands can be strengthened, either by configurational modulation, involving cytoskeletal changes and protein kinase C activation, or by increased local concentration of divalent cations (Mn$$^{+2}$$, Mg$$^{+2}$$), or by the autocrine secretion by PMNs of binding factors, such as integrin modulating factor [24]. A third mechanism of modulation of EC and PMN recognition involves platelet activating factor (PAF), and is implicated in thrombin or histamine activation of endothelium [39]. Under these conditions of activation, ECs synthesise and express PAF on their cell surface [23]. Together with P-selectin, PAF then binds to its receptor present on PMNs and induces an increase in Mac-1 expression, as well as a rapid but transient (30 min) enhancement of PMN adhesiveness to ECs.

E- and L-selectins represent an additional group of molecules implicated in the regulation of leucocyte-EC interaction [24]. Thus, E-selectins are only expressed by activated ECs and bind to L-selectins and probably other PMN surface ligands. The recruitment of E-selectins at the EC surface is at least one of the mechanisms involved in IL-1 and TNF-$$\alpha$$-induced enhancement of PMN adherence to ECs [39]. Increased adherence begins one hour after IL-1 stimulation, in contrast to the short-lived effect of thrombin or histamine stimulation involving P-selectin and Mac-1. This strongly suggests the possibility of a sequential regulation of PMN-EC interactions.

Besides its direct adhesive properties, L-selectin, in its soluble form, has been found to upregulate CR3 expression on PMNs, thereby acting as a signal transducer [40]. This dual effect of L-selectin could explain why this adhesion molecule is equally important for PMN adherence to ECs as the $$\beta_2$$-integrins [41]. L-selectin expression on the cell surface can also be upregulated under appropriate PMN activation. Shortly after upregulation and binding to ECs, L-selectin undergoes proteolytic cleavage, followed by its release in the extracellular compartment, where its function remains to be defined.

Despite some uncertainties in the adherence process of leucocytes to ECs, this interaction represents an area of research where considerable information has accumulated, with a better understanding of this crucial prerequisite step of inflammation [42]. At this stage, it is tempting to speculate on the potential sequence of events in response to an inflammatory stimulus. Thus, in resting conditions, PMNs are confined to the intravascular compartment, part of them forming the margination pool, mostly in the postcapillary venules. These physiological conditions could involve a limited interaction between $$\beta_2$$-integrins and ICAM-1 expressed at a low level. Shortly after stimulation, L-selectins are recruited to the PMN surface and bind to counter receptors (E-selectin and other not yet defined ligands), inducing an enhanced interface with EC. This could result in the "rolling" of PMNs on the EC surface. This slow motion of PMNs on the endothelial layer, together with changes in blood flux, could initiate and enhance the interaction between ECs and PMNs. Further stimulation induces the shedding of L-selectins and the increased expression of $$\beta_2$$-integrins and ICAM-1, respectively, on PMNs and ECs leading to a more intimate contact between the two cells. In addition, upon stimulation e.g. by IL-1, ECs express on their surface E-selectins not present in resting conditions. Other factors, such as PAF, also contribute to reinforcing the binding of PMNs to ECs through configurational changes of P-selectins and induction of Mac-1 upregulation.

Once immobilized, PMN can become polarized and the transmigratory process can take place. In the induction of PMN transmigration, L-selectin appears critical, since the shedding of L-selectin is required for the extravasation process to start. The majority of the mediators involved in the adherence process are also implicated in the regulation of PMN migration [43]. It seems, therefore, reasonable to assume that the concentration of these mediators is likely to be critical in the determination of which of the two activities, adherence or migration, is favoured. For C5a, it has been demonstrated that high concentrations increase PMN adherence and decrease migration, whilst the reverse is observed for concentrations below 1 nM [44]. Moreover, it has been shown that PMN migration occurred along a gradient of C5a molecules bound to a substrate (haptotaxis), rather than from the lowest to the highest concentration of C5a (chemotaxis) [45]. These substrate-bound molecules create a pathway for the PMNs through the endothelial barrier, the interstitial tissue and, potentially, the epithelial cells. The different structures encountered by PMNs during migration can also influence their motile behaviour. For example, the potency of various chemoattractants was shown to vary in vitro according to the transgressed barrier, with LT$$\beta$$$_2$ inducing better trans-endothelial migration than FMLP, and the reverse for transepithelial passage [46].

PMN adherence to epithelial cells has also been demonstrated, with increased attachment to epithelial cells previously incubated with cigarette smoke or infected by respiratory viruses, such as parainfluenza virus type 2 [47]. ICAM-1 expression and upregulation on epithelial cells appears to play a predominant role in the regulation of this adherence process. The modulation of the interaction between epithelial cells and PMNs could have relevant clinical implications in respiratory infections, where PMN adherence could facilitate their microbicidal activity. By contrast, in chronic bronchitis, increased adherence of PMNs to activated epithelial cells could induce uncontrollable neutrophil-mediated cytotoxicity, leading to epithelial damage.

A number of mediators, such as biologically active phospholipids, cytokines and bacterial products, have been reported to influence PMN motility. However, discrepancies have been observed between in vivo and in vitro studies. These discrepancies appear to reflect the complex interactions between cells, mediators and matrix components involved in PMN migration towards tissues. Among the inflammatory mediators, C5a, NAP-1/IL-8, PAF, the arachidonic acid metabolites LT$$\beta$$, and 5-hydroxyeicosatetraenoic acid (5 HETE) are considered as the major chemoattractants for PMNs, at least in vitro.
TNF-α and IL-1 also induce PMN extravasation, but only in vivo. This activity is probably related to the enhancement of PMN adherence to ECs as discussed previously, and to the stimulation of other inflammatory cells to release PMN chemoattractants.

In particular, the alveolar macrophages (AMs) appear to play a pivotal role in PMN traffic to the alveoli and the distal airways [43]. Thus, AMs clear most of the occasional particles and microorganisms reaching the lower respiratory tract. The clearance involves mechanisms which do not require major cell activation, and this combined with the poor accessory cell activity of AMs prevent a permanent inflammatory and immune response in the lung. However, when AMs are overwhelmed, either by the size of the inoculum or by the type of pathogen, they can contribute to PMN recruitment, via the release of NAP-1/IL-8, LTB₄, PAF, 5 HETE, IL-1β and TNF-α (figs. 1 and 2).

In addition to these chemoattractants, AMs also release at least one low molecular weight inhibitor of PMN motility and respiratory burst [48]. This compound has recently been shown to be present in airway secretions, and its presence correlates inversely with the intensity of bronchoconstriction induced by inhalation challenge, suggesting a potential protective role in airway hyperactivity [49]. Other inhibitors of PMN migration have been described: 1) serum factors which inhibit C5a-induced PMN chemotaxis; 2) a protein initially isolated from lymphocyte culture supernatants, called neutrophil inhibiting factor, and now recognized as GM-CSF; and 3) lipoxin A₄ (LXA₄), an arachidonic acid metabolite derived from leukotriene A₄ (LTA₄) released by PMNs and further

Fig. 1. – Schematic illustration of the initial events associated with bacterial infection in the alveolar space. When the pathogens bypass the mucociliary and alveolar macrophage (AM) clearance, bacterial products and AM activation initiate the inflammatory response. Ep I and Ep II: alveolar type I and type II epithelial cell; cap: pulmonary capillary; LPS: lipopolysaccharide.

Fig 2. – Polymorphonuclear neutrophil recruitment. Alveolar macrophages (AMs), as the residential phagocytes, are activated to release a series of mediators (IL-8, LTB₄, PAF) which, together with the complement fragment C5a, induce PMN adherence and extravasation. Additional AM-derived cytokines (IL-1β and TNF-α) indirectly promote PMN migration. IL: interleukin; TNF: tumour necrosis factor; TGF: transforming growth factor; PAF: platelet-activating factor; LTB₄: leukotriene B₄; PMN: polymorphonuclear neutrophils.
metabolized by platelets through the sequential activity of 5 and 15 lipoxygenase [50–52]. Both the CSa inhibitor and LXA₄ have been detected in bronchial washings, and GM-CSF can be produced by AMs. When PMN extravasation needs to be reduced or completely blocked, the PMN inhibitors must rapidly take over the activators in order to prevent further PMN activation (fig. 3). In this context, the fate of NAP-1/IL-8, a cytokine with major effects on PMNs (fig. 4), is instructive. Firstly, when PMNs are continuously exposed to high concentrations of intravascular IL-8, they undergo surface expression and shedding of L-selectin before contact with ECs and, as a consequence, these PMNs lose their capacity to appropriately initiate their extravasation [53]. Secondly, red cells were shown to bind the majority of IL-8 present at biologically active concentration in the blood, via specific high affinity binding sites [54]. Finally, a recent study has documented the presence of circulating anti-NAP-1/IL-8 antibodies in peripheral blood [55]. Together, these mechanisms illustrate the complex network of events potentially implicated in the regulation of PMN adherence and migration.

Fig. 3. - Polymorphonuclear neutrophils (PMNs) recruited in the alveoli release their secretory products upon stimulation by bacterial polypeptides, LPS and bioactive mediators (γ-IFN, IL-6, IL-8, GM-CSF). Some mediators (IL-8, GM-CSF) implicated in the initial attraction and activation of PMNs, as well as inhibitory factors such as the alveolar macrophage-derived inhibitor of PMN (AMDCI) and nitric oxide (NO) derived from ECs can block PMN migration, providing an attractive mechanism to downregulate the inflammatory reaction after the eradication of the pathogens. γ-IFN: gamma-interferon; GM-CSF: granulocyte-macrophage colony-stimulating factor; EC: endothelial cell. For further abbreviations see figures 1 and 2.

Fig. 4. - NAP-1/IL-8 represents a major stimulus for polymorphonuclear neutrophils (PMNs). As illustrated, NAP-1/IL-8 can interact with PMNs at several levels, including adherence, migration and degranulation. Part of these effects could be mediated by the increase in transmembrane Ca²⁺ flux. NAP-1/IL-8: neutrophil-activating peptide-1/interleukin-8; ↑: increase; ↓: decreased.
**ROLE OF POLYMORPHONUCLEAR NEUTROPHILS**

Secretory product and biological functions

After extravasation, and upon stimulation, PMNs release a series of secretory products able to react with pathogens and host tissue cells and matrix. These PMN-derived products are mostly stored in lysosomal granules. These granules are subdivided into azurophil (primary) and specific (secondary) granules, according to their morphology and content (table 2). After PMN activation, the granules migrate to the cell membrane and the fusion with the membrane initiates the exocytosis, followed by the release of the granule content into the extracellular medium.

In addition to these intracellular products, PMNs can undergo a respiratory burst and release substantial amounts of reactive oxygen metabolites, including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hypochloride, and hydroxyl radical (OH•) [56]. The one electron reduction of molecular O₂ generates O₂⁻, and the reaction is mediated by the nicotinamide-adenine-dinucleotide-phosphate (reduced form) (NADPH) oxidase complex. Most of O₂⁻ produced by PMNs is enzymatically converted to H₂O₂ by a Cu-Zn superoxide dismutase. PMNs can either detoxify H₂O₂ to H₂O through the glutathione redox system, or produce hypochallic acids in the presence of halide and myeloperoxidase. PMNs contain substantial amounts of myeloperoxidase in their azurophil granules, and using Cl as a substrate release large quantities of HOCI. Hydroxyl radicals (OH•) are formed in the Haber Weiss reaction, by the combination of O₂⁻ and H₂O₂ in the presence of Fe²⁺. Together with H₂O₂ and HOCI, OH• represent potent oxidative metabolites, accounting for a large part of PMN microbiical activity. In addition, PMNs also generate long-lived oxidants (half-life of 8 h) such as N-chloramines, capable of inducing a prolonged oxidative reaction.

The role of these oxygen metabolites in vivo is best emphasized in patients with chronic granulomatous disease (CGD) characterized by a defect in the respiratory burst of phagocytes [57]. The disease is inherited, either X-linked (most common form) or autosomal recessive, and is caused by an impaired function of the NADPH oxidase complex. Patients with CGD present in their childhood with severe, frequent and prolonged infectious problems, in particular pneumonia and soft tissue infections, with cold abscesses. The organisms most commonly isolated from infected sites are *Staphylococcus aureus*, *Pseudomonas spp.* and *Nocardia sp*. In contrast to patients with CGD, patients with myeloperoxidase deficiency suffer only minor infectious episodes, suggesting that in these patients PMNs can compensate with other microbiical mechanisms.

Several other secretory products of PMNs can contribute to killing the pathogens. Firstly, lysozyme, present in both azurophil and specific granules, can induce the hydrolysis of glycosidic linkages in bacterial cell walls. Lactoferrin, a protein stored in PMN specific granules, is also considered to have a bacitracidical activity, partly, but not exclusively, dependent on its iron binding capacity [58]. More recently, a group of endogenous antibiotic proteins, stored in azurophil granules, has been found to have bactericidal properties [59]. This group includes cathepsin G and the defensins (human neutrophil peptides (HNP-1 to 4), cationic antimicrobial protein (CAP) 37, azurocidic and bacitracidical/permeability increasing protein (BIP). Although the precise contribution of each individual molecule to the in vivo antimicrobial activity of PMNs remains to be established, it is possible that these antibiotic proteins provide alternative bacitracidical agents in anerobic conditions.

Both azurophil and specific granules contain proteolytic enzymes active on fibres and proteoglycans of the interstitial matrix. Neutrophil elastase, a neutral serine protease, represents quantitatively the major azurophil granule component (3 µg·10⁶ cells). The activity of neutrophil elastase is not restricted to elastin fibres, since type I, II, III and IV collagen, glycoproteins such as fibronectin and proteoglycans, can also be cleaved by...
this neutral protease [43]. In the lung, PMN elastase has been implicated in the pathogenesis of destructive diseases such as emphysema associated with α1-protease inhibitor (α1-PI) deficiency [60]. In addition to its proteolytic properties, PMN elastase can influence, at least in vitro, endothelial cell functions. Thus, detachment of endothelial cells from their matrix support, as well as cell lysis, can be induced by PMN elastase [61, 62]. Also, several functions of epithelial cells appear to be affected by PMN elastase. More specifically, a decrease in the frequency of ciliary beats on ciliated cells, an increased secretion of mucus, and an enhanced expression of mRNA transcripts for IL-8 in transformed epithelial cells have been documented in the presence of PMN elastase [63]. If these changes occur in vivo, they would provide a role for this serine protease, not only in emphysema but also in bronchial diseases, such as chronic bronchitis, asthma and cystic fibrosis. Moreover, when complexed to α1-PI (its major antiprotease) PMN elastase is chemotactic for PMNs, illustrating the difficulty of globally evaluating the interaction between proteases and antiproteases. Recently, a serine protease, different from elastase and present in azurophil granules, was shown to degrade elastin fibres, and was identified as proteinase 3 [64]. At acidic pH (6.5), the digestion of elastin by proteinase 3 and elastase are similar.

In addition to an elastolytic activity, PMNs can secrete a collagenase, preferentially active on type I as compared to type III collagen, and stored in its latent form in specific granules [65]. The secondary granules also contain two enzymes with specific proteolytic activity on matrix components: heparanase inducing the hydrolysis of heparan sulphate, a proteoglycan from the basement membrane, and gelatinase, a metalloproteinase with specific activity on type V collagen, a major constituent of the basement membrane [66, 67]. The release of these enzymes can be selective, and can be achieved with low concentrations of stimuli, suggesting that they can contribute to facilitate the PMN migration through the interstitial matrix, without major structural changes in tissues. Although gelatinase has been reported to be co-localized with lactoferrin in the specific granules, other studies suggest that gelatinase is stored apart from azurophil and specific granules, in intracytoplasmic vesicles. The content of these vesicles has recently been characterized to be mainly albumin, together with other plasma proteins, such as transthyretin, IgG and tranexatin [68]. These proteins are internalized, and not synthesized by PMNs. Alkaline phosphatase, cytochrome b558, and CR1 are also stored in these vesicles. Interestingly, the exocytosis of intracytoplasmic vesicles is induced by concentrations of chemotactic factors lower than those required for degranulation of azurophil and specific granules. Moreover, these vesicles were recently shown to contain Mac-1 (CD11 b/CD18), providing one mechanism by which PMNs can rapidly recruit and increase the expression of β2-integrins on their membrane. This process probably plays a role when PMNs are exposed to sustained stimulation of chemotactic factors [69].

PMNs also contribute to the pool of inflammatory mediators, via the release of bioactive lipids, essentially LTB₄, and PAF, and the secretion of cytokines (IL-1 and NAP-1/IL-8). The production of IL-1α and β by PMNs has been convincingly demonstrated, as well as the concomitant release of an IL-1 inhibitor [70–72]. Recently, PMNs were found to synthesize and secrete significant amounts of NAP-1/IL-8 in response to phagocytosis [73].

With their arsenal of secretory products, PMNs appear as first line effector inflammatory cells, potentially activated by various stimuli. Thus, the activation of the respiratory burst and the induction of degranulation in PMNs can be achieved in vitro after exposure to several stimuli, including bacterial peptides, immune complexes, complement fragments, GM-CSF, LPS, IL-6, IL-8, TNF-α, LTB₄, and PAF [74]. However, the presence of inhibitory mechanisms is equally important, since they can prevent an inadequate and prolonged activation of PMNs, which could lead to tissue damage. Such inhibitors of PMN function have been identified as secretory products derived from ECs (prostacyclin, adenosine and nitric oxide), from AMs (prostaglandin E₂, alveolar macrophage derived PMN inhibitor) and from platelets (LXA₃) [43, 75].

**PMNs in respiratory diseases**

**Neutrophils and antimicrobial defences**

PMNs are primarily implicated in the defence of the respiratory tract, where their protective role contributes to prevent bacterial growth and to eradicate the infectious process when pathogens proliferate [76]. PMNs are therefore considered as major cellular components in the defence of the lung against bacterial and fungal infection. Animal models of infection, as well as clinical studies, strongly suggest that the ability to remove bacteria from the respiratory tract depends essentially on the number of PMNs present in the lesions [77]. It is well recognized that patients with marked neutropenia or severe defects in PMN function often present with respiratory problems, such as recurrent pneumonia, and other types of life-threatening bacterial and fungal infections [78]. However, in these patients, infections do not always occur, suggesting that other host defence mechanisms are involved and could compensate for the decrease in PMN number or function. Several acquired and congenital diseases, characterized by abnormalities of PMN function, have been reported [57]. Despite recent progress in the understanding of PMN functions, the biochemical or morphological basis of the defects associated with these diseases are often complex, and the relevance of the neutrophil defect to the presentation of the disease is sometimes unclear. As already discussed, the adherence properties of PMNs influence their transendothelial migration and, indirectly, their antibacterial role at the sites of infection. In this context, CR3 deficiency, a rare autosomal recessive disorder, affects the adherence-related functions of PMNs. In CR3 deficient patients, PMNs exhibit a decreased capability to aggregate, a
decreased adherence to ECs, a poor phagocytosis of opsonized microorganisms, defective spreading, a decreased spontaneous mobility and chemotaxis. This results in an inappropriate inflammatory response and life-threatening bacterial and fungal infections [57]. Additional congenital defects in PMN function, associated with recurrent infections, include abnormal respiratory burst associated with the CGD as already discussed previously, and the Chédiak-Higashi syndrome, characterized by a lysosomal dysfunction [78].

The acquired PMN deficiencies largely outnumber the congenital disorders, and include mostly neutropenia, associated with myeloproliferative diseases and induced by cancer chemotherapy and radiation therapy [43]. Both Gram-positive and Gram-negative infections are observed in patients with neutropenia, although the latter are more frequent. This is in agreement with animal studies supporting a predominant role for PMNs in models of Klebsiella and Pseudomonas pneumonia. For Gram-positive organisms, such as Staphylococcus aureus, small inoculi are generally cleared by AMs whilst larger numbers of pathogens require the cooperation of PMNs.

**Neutrophils and lung injury**

In addition to their role in infectious disorders, PMNs are implicated in the pathogenesis of bronchopulmonary diseases, such as chronic bronchitis, asthma, cystic fibrosis, the adult respiratory distress syndrome, idiopathic pulmonary fibrosis, collagen vascular disorders and emphysema. The oxidative and proteolytic mediators released by PMNs to kill microorganisms can also act on adjacent host tissues and, therefore, be involved in the pathogenesis of a number of noninfectious diseases of the lower respiratory tract. Virtually absent in the lung parenchyma and airways of healthy subjects, PMNs can be attracted from the pulmonary circulation by PMN chemotactic factors. Some of these factors have been found to be increased in the airways of patients with parenchymal lung diseases associated with increased alveolar PMN counts.

**Smoking, chronic bronchitis and emphysema.** In cigarette smokers, there is an increase of both peripheral blood and BAL PMNs in comparison with nonsmoking controls [79]. It is unlikely that tobacco smoke directly attracts PMNs to the lung, since no chemotactic activity for PMNs is observed in vitro with smoke extracts. However, alveolar macrophages from nonsmokers in the presence of tobacco smoke increase their release of chemotactic factors in vitro. The consequences of PMN accumulation in the airways of smokers are difficult to interpret. Thus, the higher number of PMNs in the airways could increase the antimicrobial defence but could also impair bronchial cell function. These functional changes could prevent the normal response to the bacterial challenge [80]. In vitro studies on the effect of cigarette smoke on PMN function are controversial, some authors reporting enhanced functions, others suggesting depressed reactivity [81–83]. Moreover, the relevance of active smoking on PMN function in vivo remains speculative.

In chronic bronchitis, PMN counts in the airways are increased but the changes of PMN function are also a subject of controversy. Some authors report that PMNs from chronic bronchitis patients have a defect in chemotaxis, phagocytosis and killing of Candida [84]. Also, the oxidative metabolism of PMNs has been reported to be significantly reduced, and a constitutional defect in neutrophil response could be an underlying cause for the increased susceptibility to infections associated with chronic bronchitis. Other authors report that the chemotactic responses of PMNs are enhanced [85]. The latter observation provides a mechanism for the increased recruitment of PMNs to the airways of these patients [86]. Whether this increase represents a cause or a consequence of the disease is unknown. However, through the release of oxygen radicals and proteases, PMNs could contribute to inflammation, bronchial damage and mucus hypersecretion, three characteristic features in chronic bronchitis.

Emphysema is believed to be caused by an impaired balance between proteolytic and antiproteolytic activities in distal airways and alveoli [87]. This is best illustrated in patients deficient in α1-PI, the major antiprotease against PMN elastase. These patients develop emphysema early in life. However, α1-PI deficiency accounts for only a minority of cases of emphysema. By contrast, cigarette smoking represents a major risk factor, although individual susceptibility is likely to play an important role, since not all heavy smokers will eventually have emphysema [88]. One mechanism contributing to the exposure of smokers to the risk of emphysema is the retention of PMNs within the pulmonary microvasculature, associated with active smoking. This slower wash-out of PMNs could potentially lead to lesions of lung cells and matrix [89]. In addition to its role on PMN recruitment and activation, the cigarette smoke contains various oxidants capable of damaging epithelial and endothelial cells and inactivating α1-PI via the oxidation of its protease binding site. This could result in unrestrained PMN elastase activity, leading to interstitial matrix degradation. Moreover, the intimate binding of PMNs to tissue could prevent the access of antiprotease macromolecules to the site of proteolytic burden. This is also likely to be critical for the outcome of injurious processes.

**Asthma.** Although PMNs are probably not the dominant cells in asthma, they may contribute to the inflammatory process underlying the disease [90]. Thus, PMNs are increased in bronchial biopsies, as well as in airway washings, from asthmatic patients. Other inflammatory and immune cells implicated in asthma, such as mast cells, eosinophils and lymphocytes, represent major sources of PMN chemotaxins. Among these chemotaxins, LTB4 and PAF have been found to be present in BAL fluid from asthmatics [91, 92]. Moreover, chemotactic activity for PMNs can also be detected in the serum after antigen-induced late phase asthmatic reaction [93].
PMNs are believed to play an important role in several models of airway hyperreactivity. Thus, bronchial hyperreactivity induced by chemicals (toluene diisocyanate) or by vegetable extracts (cotton bract) are associated with PMN activation [94, 95]. In the cotton bract model, PMN functions appear to correlate with the degree of bronchoconstriction. More recently, an inhibitory activity for PMN motility and respiratory burst was found to parallel the low responsiveness of the airways of normal subjects to the cotton bract challenge [48]. To further support a role for PMNs in asthma, Metzger et al. [96] reported that O₂ production by blood PMNs from asthmatics correlates with the severity of bronchial constriction [96]. A similar role for oxygen radicals has also recently been suggested in nocturnal asthma, although the contribution of PMNs to the O₂ production by airway cells from asthmatics remains to be defined [97].

Cystic fibrosis. Cystic fibrosis (CF) is a genetic disorder caused by an impaired permeability for chloride ions in epithelial cells, leading to progressive and destructive airway disease and bronchiectasis in children and young adults [98]. The disease is characterized by chronic airway infection and inflammation, invariably dominated by PMNs. The fluid lining the respiratory epithelium contains large numbers of PMNs and active PMN elastase. PMN elastase can damage epithelial cells by direct toxic effect, can hinder normal host defence by interfering with ciliary clearance, increasing mucus production, cleaving immunoglobulins and complement, and by impairing phagocytosis and killing of Pseudomonas aeruginosa by lung phagocytes [99]. Moreover, PMN elastase appears to be the dominant signal capable of inducing the expression of the IL-8 gene, and the release of IL-8 by the respiratory epithelium [63]. Thus, IL-8 has been detected in airways secretions from CF patients, and is likely to contribute to the recruitment of PMNs [100]. Until now, the treatment of CF mostly consisted in preventing recurrent infections in the respiratory tract. However, the recent recognition of the CF regulator gene, and the progresses made in gene therapy, could change the prognosis of this lethal genetic disease in the reasonably near future [101, 102].

Cancer. The ability of PMNs to kill in vitro tumour cells by oxidative and non-oxidative mechanisms, such as defensins and cathepsin G, is well-documented [103]. By contrast, the increased secretion of protease by PMNs can contribute to the invasive behaviour of cancer cells, and local proteolysis can be enhanced by surface receptors for proteases on the tumour cells. PMNs are often the first host cells to infiltrate the tumour, and can elicit the influx of secondary effector cells, such as macrophages, natural killer (NK) cells and cytolytic T-lymphocytes, into the tumour bed, and possibly participate in their activation. A question concerning the clinical relevance of this phenomenon is whether stimuli are present in vivo and can activate the tumoricidal activity of PMNs. Recent studies suggest that cytokines such as γ-interferon (IFN), IL-1, TNF-α and CSFs could provide these required activation signals. Because these recombinant molecules are now available for experimental therapy, activation of tumoricidal PMNs in patients may become feasible.

Adult respiratory distress syndrome (ARDS). Although ARDS can occur in neutropenic patients, several lines of evidence suggest that PMNs are involved in the initiation and the severity of acute lung injury in ARDS patients with normal PMN supplies. In most individuals with ARDS, there is a marked increase of PMNs in the alveolar spaces, where they often account for 85% or more of the total cells recovered by BAL [104]. PMN extravasation, by itself, probably does not induce alveolar damage, since bacterial pneumonia associated with high alveolar counts of PMNs generally clear, without major parenchymal lesions. However, lung injury probably occurs when PMNs migrating into the alveoli are stimulated to secrete elastase and myeloperoxidase. Thus, PMN elastase, collagenase and myeloperoxidase concentrations in BAL were reported to be increased, and to correlate with the severity of gas exchange abnormality in the lung of ARDS patients [105]. When stimulated by complement fragments or phorbol esters, phagocytes, including PMNs, can produce toxic oxygen metabolites, and H₂O₂ has been detected in exhaled air from ARDS patients [106]. Other studies have demonstrated that endothelial cells, initially injured by free radicals increase their adhesiveness for PMNs and become more susceptible to further injury by PMNs. Additional evidence for an important role of oxygen free radicals in endotoxin-induced lung vascular injury is provided by the observation that N-acetylcysteine, a free radical scavenger, diminishes the degree of microvascular injury [107]. Numerous studies have implicated oxygen metabolites in the pathogenesis of acute lung injury, and PMNs are considered as major sources of free radicals. Recently, several PMN chemotaxins, such as LTB₄, TNF-α and IL-8, released mainly by activated macrophages, were found in the epithelial lining fluid of patients with ARDS [108, 109]. This explains why PMNs, normally absent, are attracted from the peripheral blood to the respiratory tract. In particular, the major influx of PMNs into the alveolar space observed in ARDS is associated with high levels of NAP-1/IL-8 in BAL [110]. Moreover, NAP-1/IL-8 appears to be the major PMN chemotactic activity present in BAL from these patients. As a clinical correlate, highest levels of NAP-1/IL-8 in BAL were generally found in patients who eventually died. More importantly, a preliminary study reported that in patients at risk for ARDS, those with high levels of BAL NAP-1/IL-8 were more likely to develop ARDS [111]. In addition to its chemotactic activity, IL-8 activates PMNs, induces the respiratory burst, and the release of proteases and LTB₄ [112]. In Gram-negative sepsis, endotoxin contributes largely to the accumulation of PMNs responsible for lung injury, through the release of cytokines active both on PMNs and ECs. The accumulation of PMNs is followed by ultrastructural evidence of pulmonary endothelial injury, and subsequent increased lung vascular permeability. These lesion can be prevented by PMN depletion.
Wegener's granulomatosis. The discovery of antibodies directed against neutrophil products (ANCA) in serum of patients with vasculitis, such as Wegener's disease, have at least suggested a role for PMNs in these disorders [113, 114]. Briefly, three groups of autoantibodies have been described, according to their pattern of immunofluorescence staining [115]. The first group essentially stains the perinuclear area (pANCA) and primarily recognizes myeloperoxidase and to a lesser extent elastase and lactoferrin. The second group of antibodies (cANCA) stains the cytoplasm of PMNs and has the proteinase 3 and probably the natural antibiotic CAP57 as targets. These cANCA are frequently associated with Wegener's granulomatosis. The third group of autoantibodies (xANCA) with a mixed (cytoplasmic and perinuclear) staining pattern recognizes cathepsin G, and is mostly present in ulcerative colitis.

As recently reviewed, circulating cANCA have a sensitivity of 75% for active Wegener's disease, and this sensitivity increases to 90% when pANCA are also present [116]. The specificity of cANCA for Wegener's disease is close to 100%. Beyond their role as diagnostic tools, ANCA have also been shown to induce the release of several mediators (TNF-α, O2•-, proteolytic enzymes) which are toxic for endothelial cells. Further investigations will probably elucidate the contribution of these autoantibodies to vasculitis more precisely.

Interstitial lung diseases

The presence of PMNs infiltrating the lung parenchyma is generally associated with injurious and fibrotic disorders, such as idiopathic pulmonary fibrosis (IPF) and collagen vascular disorders (CVD). Thus, enhanced chemotactic activity for PMNs is detected in BAL fluid from both IPF and CVD patients [117, 118]. Collagenase activity is also present in BAL fluid from these patients, as well as PMN elastase [119, 120]. It is recognized that some CVD patients without overt pulmonary involvement can also have elevated PMN counts in their BAL [121]. The significance of this alveolar neutrophilia is unknown. However, only CVD patients with I LD have detectable levels of PMN elastase in their BAL, suggesting that in CVD patients without I LD, either the products of PMN granules are not secreted, or these products are rapidly neutralized by antiproteases.

PMN accumulation in the alveolar space is a common hallmark in IPF patients [122]. Thus, the number of BAL PMNs correlates with the increased expression of mRNA for IL-8 by alveolar macrophages in these patients, providing at least one mechanism of PMN recruitment [123]. Although PMNs can contribute to the lesional changes observed in fibrotic ILD, via the release of proteases and/or oxidants, as illustrated by increased levels of oxidized glutathione and methionine residues in BAL [124, 125], their role in fibrotic changes is unclear. In particular, there is little evidence that PMNs release major fibroblast growth factors or promote the production of collagen fibres. Moreover, the prognostic value of high numbers of PMNs in BAL from IPF patients is uncertain. For example, in smoking patients with IPF, a persisting high number of BAL PMN is not associated with functional deterioration [122]. This is in contrast with the presence of eosinophils in BAL from IPF patients, usually associated with a poor prognosis, and the presence of high lymphocyte counts frequently associated with better prognosis [126].

Hypersensitivity pneumonitis (HP) is characterized by a delayed type reaction to organic or chemical antigens, and by an intense immune response in the lung. The dominant cells are T-lymphocytes, with a majority of T-suppressor/cytotoxic cells (CD8). However, PMNs are often increased, and can account for more than 5% of BAL cells. This increase in PMNs is more striking when BAL is performed in HP patients shortly after specific antigen challenge, probably in the context of an acute inflammatory response [127].

The presence of PMNs in lungs from patients with pulmonary sarcoidosis is not a usual feature in a disease characterized by the infiltration of the interstitium by granuloma, formed by a mixture of epithelioid and giant cells and T-lymphocytes. Although the presence of PMNs infiltrating the interstitium has originally been associated with chronic and fibrotic disease, moderate increases in BAL PMN counts have also occasionally been observed in more recent cases of sarcoidosis and, therefore, the relevance of this increase in non-fibrotic forms of the disease is uncertain [128, 129].

Finally, an increased number of PMNs has been reported in bronchiolitis obliterans with organizing pneumonia (BOOP), an interstitial lung disease characterized by focal distortion of distal airways and alveoli by granulomas. An increase in BAL lymphocytes represents the dominant picture, although PMNs, eosinophils and mast cells, are also increased, but to a lesser extent [130].

In summary, PMNs have frequently been associated with injurious and fibrotic forms of ILD. However, it remains to be established to what extent these phagocytes contribute to perpetuation of the damage, and whether they trigger the fibrotic reaction.

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ROLE OF POLYMORPHONUCLEAR NEUTROPHILS


