Toll-like receptor (TLR)-based networks regulate neutrophilic inflammation in respiratory disease

I. Sabroe and M.K.B. Whyte

Academic Unit of Respiratory Medicine, Section of Infection, Inflammation and Immunity, School of Medicine and Biomedical Sciences, L Floor, Royal Hallamshire Hospital, University of Sheffield, Sheffield S10 2JF, U.K.

Abstract

The neutrophil is a crucial early defence against microbial infection, but neutrophilic inflammation can result in devastating acute and chronic inflammatory diseases. In the lungs, the neutrophil is a principal part of the pathology of the acute respiratory distress syndrome, and its activation may also be of substantial importance in chronic obstructive pulmonary disease and some forms of asthma. Induction of neutrophil recruitment in response to microbial attack requires activation of TLR (Toll-like receptor)-based signalling pathways and the concerted actions of multiple cell types, including sentinel cells such as monocytes and macrophages acting together with tissue cell types such as the epithelium or smooth-muscle cell. The present review describes some of these networks and the resulting potential for their targeting in respiratory disease.

Introduction

Current anti-inflammatory strategies targeting respiratory disease are often effective modulators of tissue cell, lymphocyte and eosinophil function, but are relatively ineffective in their ability to target the neutrophil. The ARDS (acute respiratory distress syndrome) comprises an acute neutrophilic inflammatory process resulting in a mortality of 40–60%, which is poorly responsive to anti-inflammatory therapies such as steroids [1–4]. Commonly triggered by local or systemic microbial infections, ARDS represents one of the most severe acute neutrophilic inflammatory diseases known. Neutrophils also appear to play important roles in acute exacerbations of asthma [3,5–10], and in at least some patients with fatal or near-fatal asthma, the neutrophil may be the predominant inflammatory cell found in the lungs [9,11,12].

Recruited in response to microbial stimuli, the principal role of these phagocytes is to control bacterial infections. Neutrophilic inflammation is also a feature of chronic inflammatory lung disorders, including asthma and COPD (chronic obstructive pulmonary disease) [3,10,13,14]. The pathological role of neutrophils in these diseases remains the subject of considerable debate. Recent studies have shown that asthma is a disease comprising several phenotypes [10], and patients with neutrophilic variants appear to have less response to steroid therapies [15]. COPD is associated with a chronic neutrophilic infiltrate of lung tissues and airways. The potential of neutrophil-derived proteases and oxidant species to drive the pathology of COPD has long led to a conviction that the neutrophil is likely to be an important contributor to this disease process [16].

Thus the neutrophil represents a key cell responsible for clearance of bacterial infections. It is recruited early in inflammation, but recruitment may continue over months or years in chronic disease states, and excess or inappropriate neutrophilic recruitment and activation may contribute powerfully to tissue damage. The mechanisms by which microbial and other signals cause neutrophilic recruitment and activation are therefore potential therapeutic targets.

TLR (Toll-like receptor) signalling and neutrophilic inflammation

Induction of neutrophil recruitment to a site of infection requires local generation of chemoattractants. It has long been known that many cells respond to microbial stimuli such as LPS (lipopolysaccharide), lipoproteins and flagellin by the generation of neutrophil-recruiting chemokines such as IL-8 (interleukin-8). Microbial agonists are sensed by a range of PRRs (pattern-recognition receptors), foremost among which is the TLR family [17]. Within the airway, many cell types have now been shown to express members of this receptor family, including epithelial cells [18–24], fibroblasts [25], airway smooth muscle [26,27], and endothelial cells [28,29]. In addition, airways undergo surveillance for pathogens and damage by bone marrow-derived cells including the AM (alveolar macrophage), mast cell and dendritic cell [30]. To date, most studies using in vitro approaches have focused on the individual responses of these cells to microbial agonists, but it is now becoming clear that responses to microbial stimuli in environments such as the lungs are mediated by complex networks [31].
Cell networks regulate inflammatory responses in the lungs

In order to reveal whether tissue or leucocyte TLRs are crucial to induction of inflammation, several groups have exploited chimaeric mice, in which bone marrow is transplanted from knockout mice to wild-type mice of the same strain, or vice versa. Mice in which bone marrow, and hence circulating leucocytes, are TLR4-deficient, but whose tissue cells are TLR4 wild-type, still show neutrophil recruitment to the lungs after a systemic LPS challenge [29]. In contrast, if LPS is administered to the lungs rather than the circulation, successful induction of inflammation will be dependent on TLR4 expression on leucocytes (particularly macrophages), rather than tissue cells [32]. Using a similar strategy, but targeting MyD88 (myeloid differentiation factor 88), reveals a more complex picture: effective neutrophil recruitment to the lungs after local challenge in this setting is impaired when either tissue cells or bone marrow cells are MyD88-deficient [33]. These results illustrate the complex interaction between tissue cells and leucocytes in the regulation of inflammatory responses. Developing these concepts to an understanding of human disease is hampered, however, by the difficulties in translating elegant model work in the mouse to new therapies for human diseases [31], and we have recently adopted other strategies to try and bridge the divide between the mouse and the diseased human.

Dissection of inflammatory networks in vitro

In order to investigate the mechanisms by which TLR signalling might induce neutrophilic inflammation in human lung disease, we established models employing simple co-cultures of primary human tissue cells and leucocytes. Initially, we focused on airway smooth muscle, a cell type with marked synthetic capabilities and with multiple roles in cytokine production, inflammation and airway remodelling in asthma [34]. We observed that primary human ASMCs (airway smooth-muscle cells) showed little TLR4 expression, and were relatively unresponsive to TLR4 agonists [26] (although this has not been a universal finding [27]). Addition of very small numbers of human monocytes to ASMCs enabled a profound synergistic response to purified LPS, resulting in the production of a range of cytokines heavily implicated in neutrophilic (and allergic) inflammatory processes [26]. Strikingly, this mechanism appears to be of generic importance. Pulmonary epithelial cells are held to be an LPS-responsive, TLR4-expressing cell [19,23], with this LPS response presumably forming an important component of the innate immune system in the lungs. We observed that, even for LPS-responsive cells such as the epithelial cell, addition of small numbers of monocytes to epithelial cells again profoundly amplified inflammatory responses to LPS [35]. Monocytes were similarly able to substantially amplify inflammatory responses in co-culture with either primary endothelial cells or vascular smooth-muscle cells [35]. Such co-culture models have been employed previously to demonstrate that monocytes amplify prothrombotic responses to LPS (via production of TF (tissue factor) from endothelial cells [36]), and can potentially enhance recruitment of other cells through the up-regulation of adhesion molecules such as VCAM (vascular cell adhesion molecule) [37].

We observed that signalling between monocytes and tissue cells was dependent not on cell–cell contact but on the release of a soluble mediator. TNFα (tumour necrosis factor α) has a principal role in early sepsis, yet production of TNFα is actually reduced in leucocyte/endothelial cell co-cultures, potentially via a mechanism involving inhibition of monocyte responses by secretion of calmodulin from endothelial cells [38]. We found that production of IL-1β by monocytes was essential to activation of co-cultures [26,35]. Importantly, while blockade of IL-1 using IL-1ra (IL-1 receptor antagonist) resulted in inhibition of co-culture activation in response to LPS, the levels of IL-1β generated by monocytes were insufficient to account for the level of co-culture activation seen [26]. These results place IL-1 as a crucial upstream initiator of inflammation, but suggest that other factor(s) play important roles in amplifying the subsequent response. These observations may help to resolve some of the complexities described in the experiments above using chimaeric mice. Mice that are bone marrow-TLR4+/− but tissue cell-TLR4+/+ show impaired responses to pulmonary delivery of LPS [32], while deficiency of MyD88 in either cell compartment impairs LPS responses [33]. Since MyD88 is essential for both TLR4 and IL-1 signalling [17], these results suggest that in mice that are marrow-MyD88−/− and tissue cell-MyD88+/+, responses to LPS are impaired through defective TLR4 signalling in the leucocyte compartment. In contrast, in mice that are marrow-MyD88+/− and tissue cell-MyD88−/+, responses to LPS may be impaired because of an inability of tissue cells to respond to leucocyte-derived IL-1.

Interestingly, neutrophilic inflammation is regulated by monocytes at multiple levels. Neutrophils are short-lived cells whose function is limited by a rapid natural progression to death by apoptosis [2]. At sites of inflammation, apoptosis is delayed, allowing the neutrophils to engage effectively with pathogens over a much longer time course. Numerous mediators, including exogenous pathogen-related molecules and endogenous cytokines, have been shown to delay neutrophil apoptosis [2]. Of these, LPS was thought to be a potent stimulator of neutrophil survival, acting again via IL-1β [39], since it was apparent that LPS-activated neutrophils were themselves a source of IL-1β, which was an effective stimulator of survival in an autocrine fashion [39]. These results are somewhat contradictory to evidence that neutrophils are in fact a sink for IL-1, consuming it without responding to it, as a consequence of their predominant expression of the IL-1 decoy receptor, IL-1R1I (type II IL-1 receptor) [40]. We observed that neutrophils, when highly purified to remove all contaminating leucocytes, showed modest or absent survival responses to either LPS or IL-1β [41–43]. Small numbers of LPS-responsive monocytes were
able to allow neutrophils to survive when LPS was present [43,44] (similar results have been found by other groups investigating the regulation of eosinophil survival [45]). This survival response is not mediated by IL-1β [41], showing an important compartmentalization of the regulation of neutrophil responses to LPS.

Hollingsworth et al. [32] demonstrated that a local pulmonary response to endotoxin could be reconstituted in TLR4-deficient mice by either reconstitution of these mice with wild-type bone marrow or delivery to the lungs of wild-type AMs. Given the variable and potentially high level of contamination of our environment with microbial-derived molecules such as LPS [46,47], it is interesting that the airways are not, as far as we can tell, chronically inflamed. In part, this is because of a variety of physical defence measures, including the trapping of particulate matter in larger airways by mucus and the ability of various airway-secreted molecules such as surfactant proteins to bind and neutralize LPS [30]. In addition, function of the AM is regulated at a variety of further levels. AMs are held in a quiescent state through the actions of TGF-α (transforming growth factor-α), presented by epithelial cells on the integrin αVβ6 [48]. This inhibition is overcome by activation of macrophages by microbial stimuli [48], but AMs are relatively slow producers of IL-1β, manufacturing this cytokine over a slower time course than activated monocytes [49]. Additionally, natural regulation of chronic signalling by induction of LPS tolerance [50], which certainly occurs in smokers [50], may play roles in down-modulating responses to chronic stimulation. Once inflammation has been established, however, replacement of AMs by a more activated population of tissue and airway monocytes [51] may result in a markedly altered tissue environment with greater potential for a more IL-1-driven pro-inflammatory response to microbial infections. In addition, there is ample potential for a variety of other leucocyte cell types resident in the lungs, including dendritic cells with close relationship to the epithelium, mast cells and intraepithelial lymphocytes, to contribute to the regulation of neutrophil inflammation [30].

**Beyond LPS**

Neutrophils may also have important roles in viral infections. Neutrophil recruitment in response to respiratory viruses is well described and may play important roles in a variety of pathologies, such as respiratory syncytial virus-induced bronchiolitis [52]. Induction of antiviral responses by TLRs and other PRRs may be mediated at the level of the tissue cell, where for example TLR3, RIG-I (retinoic acid-inducible gene 1) or mda5 (melanoma differentiation-associated gene 5) activation may induce generation of neutrophil-recruiting chemokines and other pro-inflammatory mediators [22,25,27,35,53]. Human monocytes do not express functional TLR3 (in leucocytes, this is principally found in immature dendritic cells), but do express receptors for single-stranded viral RNA such as TLR8 [35,54]. Once again, we have found that co-operative responses to viral-like stimuli may be mediated by leucocytes and tissue cells in co-culture, and we observed that TLR agonists in combination may further stimulate co-cultures to activate specific patterns of immunity [35]. Once neutrophils have been recruited to tissues, they themselves have great potential to modulate the local inflammatory response. Neutrophils can accumulate in great numbers at inflammatory sites, where they may represent import sources of pro-inflammatory mediators, reactive oxygen species, and proteases. Neutrophil proteases have the ability to further modify local responses, since they can digest CD14 and decrease LPS responsiveness of some cells, but may also generate IL-8 production from epithelial cells [55] or signal via TLR4 [21,56]. Supernatants from cultured tissue neutrophils can also suppress TNFα release from macrophages [57]. Neutrophils infiltrate smooth muscle in COPD [58], where their products may cause cell activation and can also induce smooth-muscle apoptosis [59]. Products of neutrophils regulate TLR2 expression on other tissue cell types such as endothelial cells [60]. Neutrophils may also represent an important source of Th1 cytokines involved in early granuloma formation [61]. As neutrophils die, their mode of death, by apoptosis or necrosis, also has a profound effect on the inflammatory microenvironment, and may be crucial in the successful transition from an antimicrobial site of active inflammation to one in which tissue healing and restoration of normal architecture occurs [2].

As the processes of innate immunity segue into adaptive immunity and/or into an environment in which wound healing predominates, so further complex networks develop in which there is continual dialogue between multiple processes and cell types. These networks are not easily defined by conventional terminology, since at any given time in the lungs during an acute, subacute or chronic process, multiple different networks are likely to be active and in continual evolution and dialogue. We have coined the term ‘contiguous immunity’ to describe these linked immune processes [30]. The many networks that comprise pathology probably function in a fashion analogous to scale-free systems, in which determining the key points to intervene in any given system or disease may be surprisingly challenging [30,31,62]. It is likely that our relatively poor understanding of these networks is responsible for the difficulties in predicting the consequences of their targeting in human disease.

**Conclusion**

Neutrophils form a crucial and early component of the innate immune system’s response to microbial invasion. Their function is heavily regulated by TLR signalling at multiple direct and indirect levels, in intricate networks. Such networks are amenable to dissection in vitro and in vivo, potentially generating further opportunities to intervene in diseases characterized by unwanted or exaggerated neutrophil responses.

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